The Isolation and Differentiation of Adipose Derived Mesenchymal Stem Cells

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Abstract

Pathologies concomitant with old age such as arthritis, osteoporosis, and obesity diminish the quality of life for society's elderly. Recent advances in regenerative medicine have revealed that an abundant and readily available source of adult mesenchymal stem cells (MSCs), resides within adipose tissue. Previous research has demonstrated that MSCs can differentiate into multiple specialized cell types and may thus be capable of ameliorating symptoms of many age-associated diseases. However, several unique challenges currently limit the usefulness of adipose-derived MSCs in regenerative therapies. In this thesis I assessed novel ways of isolating and differentiating adipose-derived MSCs into desired cell types. Specifically, I assessed the capacity of a non-enzymatic sonication based method to the collagenase digestion based method of isolating MSCs from human adipose. Sonication yielded fewer total cells than collagenase. However, alizarin red staining revealed that cells obtained using sonication possessed a stronger osteogenic capacity than those obtained using collagenase. Additionally, oil red o staining showed that both methods produced cells capable of adipogenesis. Yet, alcian blue staining demonstrated that sonication was unable to yield cells capable of chondrogenisis. Lastly, rat adipose-derived stromal cells were cultured in medium containing stimulators of UCP1 expression in order to induce a brite cell phenotype. Following a 9 day treatment period, cultures displayed characteristic traits of brite cells such as increased intra-cellular lipid accumulation and increased mitochondrial content. However, when exposed to acute norepinephrine treatment, stromal cells did not display an increased rate of respiration. Furthermore, western blot analysis revealed that these cells did not express UCP1, suggesting that the differentiation treatment likely only

induced a partial differentiation response. Overall, the above work provides valuable insight into addressing challenges currently limiting the usefulness of adipose-derived MSCs in regenerative therapies. Future works should assess the differentiation capacity of stromal cultures depleted of non-MSC cells and stromal cells obtained using sonication in medium containing additional inducers of differentiation. Moreover, future efforts based on the brite cell differentiation method used here should assess whether extending treatment induction/differentiation periods is capable of producing brite cell phenotype.

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List of Abbreviations

BAT- brown adipose tissue

BMP- bone morphogenic protein

cAMP- cyclic adenosine monophosphate

CPC- cetylpyridinium chloride

CRE- cAMP response element

CREB- cAMP response element binding protein

DMEM- dulbecco's modified eagles media

DMSO- dimethyl sulfoxide

ECM- extra cellular matrix

FACS- fluorescence activated cell sorting

FBS- fetal bovine serum

GC- glucocorticoid

HBSS- hank's balanced salt solution

IBMX- isobutyl-methyl xanthine

IFATS- International Federation for Adipose Therapeutics and Sciences

IR- insulin receptor

IRS- insulin receptor substrate

MAP- mitogen activated phosphatase

MAPK- mitogen activated phosphatase kinase

MSC- mesenchymal stem cell

NE- norepinephrine

pCREB- phosphorylated cAMP response element binding protein

PPARγ- peroxisome proliferator-activated receptor γ

PPRE- a peroxisome proliferator activated receptor response element

SVF- stromal vascular fraction

T_3- triiodothyronine

TGFβ- transforming growth factor β

TRE- thyroid hormone response element

UCP1- uncoupling protein 1

Chapter 1: Literature Review

1.1 Introduction

Over the past several hundred years, average human life expectancy throughout the developed world has increased (1). However, greater longevity has also heightened awareness of the impact of age-related pathologies on the quality of life for society's elderly. Pathologies related to reduced bone mass through osteoporosis and loss of cartilage tissue with concomitant arthritis both increase with age. Moreover, the incidence of obesity and obesity-associated conditions such as glucose intolerance, type II diabetes mellitus, and hypertension also correlate with age. Such pathologies diminish quality of life by eliciting physical discomfort and limiting mobility (2-4). Considering this, the medical community has paid significant attention to the potential of stem cell based regenerative therapies to combat pathologies experienced in old age.

Stem cells are undifferentiated cells that divide asymmetrically into one cell that retains its undifferentiated character, and a second that can undergo one or more paths of lineage-specific differentiation (5). Research efforts towards developing regenerative therapies have revealed that populations of stem cells residing in adult tissues (e.g. bone marrow, adipose, tendon, and ligament), known as mesenchymal stem cells (MSCs), possess the capacity to differentiate along the osteogenic and chondrogenic lineages becoming bone cells (osteoblasts) and cartilage cells (chondroblasts), respectively (6). Moreover, MSCs also possess the ability to differentiate into brown fat-like cells (brite cells) which contain intracellular machinery capable of dissipating energy through mitochondrial proton leak (7, 8). Previous investigations have demonstrated that implantation of MSCs into bone or

cartilage defects enhances the repair of these tissues, while increased brown fat activity can elicit weight loss in obese animal models (9-13). Thus, MSCs possess the ability to differentiate into specialized cells capable of ameliorating multiple pathologies associated with advanced age.

For experimental and therapeutic applications, MSCs have traditionally been isolated from bone marrow. However, obtaining MSCs from marrow is invasive and painful. Conversely, adipose deposits contain MSCs, are ubiquitous, and are easily accessible in large quantities via liposuction aspiration. Moreover, adipose tissue is routinely extracted from adults undergoing liposuction surgery, and subsequently discarded (14, 15). Considering the above, the development of novel methods for isolating MSCs from adipose tissue and differentiating them into desired lineages is an import step towards developing less-invasive stem cell based therapies.

It is the aim of this literature review to: 1) Provide information regarding the isolation of MSCs from tissues and the subsequent differentiation of these MSCs into specific cell types, 2) describe the advantages of adipose derived MSCs over those obtained from other tissues, and 3) introduce literature supporting the usefulness of MSC based therapy in conferring relief from age-related diseases.

1.2 MSCs from bone marrow

During embryogenesis MSCs reside within the middle embryonic layer known as the mesoderm. The term mesenchyme is derived from the Greek meaning “middle” (meso) “infusion” and refers to the ability of MSCs within the mesoderm to spread and migrate early in development giving rise to all the body’s mesenchymal tissues: cartilage, tendon, bone, ligament, marrow, and adipose, dermis, muscle, and several

connective tissues (16). Evidence that the stroma (supportive structural framework) of adult tissue contains populations of stem cells capable of forming mesenchymal tissue originally arose from the findings of A.J. Friedenstein. By repeated pipetting, Friedenstien et al. 1970 obtained cell suspensions from guinea pig bone marrow, spleen, and bladder epithelium. The authors observed that following 9-12 days in culture, isolated cells developed into discrete fibroblast-like colonies. These cells were subsequently harvested and placed into heterotopically implanted diffusion chambers (vessels which contain cells and allow for diffusion of nutrients while barring cell migration into and out of the chamber). Using histological dyes to detect the presence of alkaline phosphatase, a marker of bone formation, the authors observed that the marrow derived cells formed bone tissue, while spleen and bladder derived cells did not (17), suggesting that stromal cells within adult marrow possess the ability to differentiate into a mesenchymal tissue.

In later decades, other works extended these observations and demonstrated that the cells identified by Friedenstein could spontaneously transform into other mesenchymal tissues including cartilage and adipose (18). The first direct evidence that bone marrow stroma contained multipotent stem cells was provided by Pittenger et al. 1999 (19). In order to obtain a purified population of marrow stromal cells, Pittinger et al isolated cells based on surface antigen expression using fluorescence activated cell sorting (FACS). They then clonally expanded these cells in order to obtain identical cultures derived from the same parental cell. These colonies were then cultured on separate plates and provided chemical inducers of adipogenic, osteogenic, or chondrogenic differentiation. Using histological stains to detect

differentiation, the authors observed that cultures derived from the same marrow cell could be differentiated into adipose, bone, and cartilage, thus demonstrating that marrow stroma contains multipotent stem cells.

1.3 MSCs from adipose

Following the discovery of MSCs in bone marrow, their potential use in regenerative medicine has been intensely pursued. Procedures used to obtain MSCs from marrow are painful and require general or spinal anesthesia. They yield relatively low numbers of stem cells (approximately 1 MSC per 10^5 tissue culture plastic adherent cells) (20). From a practical standpoint, low stem cell numbers necessitate an *in vitro* expansion step to obtain a suitable number of cells for clinical use. Such expansion is time consuming, expensive, and risks cell contamination and loss. Stem cells capable of differentiation into mesenchymal tissues have been isolated from the stroma of serval connective tissues of multiple species including, birds, mice, rats, rabbits, and humans (19, 21-26). Unlike bone marrow, adipose tissue can be obtained in large quantities through liposuction surgery (20). Yet, like bone marrow, adipose is of mesenchymal origin (27). Considering this, Zuk et al. 2001 investigated whether human adipose could be an alternative source of MSCs (28). The authors obtained human adipose from liposuction aspirate and used collagenase to liberate stromal cells from the extracellular matrix. The isolated adipose stromal cells were cultured with inducers of adipogenic, osteogenic, and chondrogenic differentiation. Zuk et al. observed that adipose stromal cells were capable of developing intracellular lipid stores, alkaline phosphatase expression, or proteoglycan expression, markers indicative of adipose, bone, and cartilage tissues, respectively. In order to determine if the isolated adipose stromal cells were

indeed stem cells, Zuk et al. 2002 examined surface antigen expression and differentiation capacity of clonogenic cultures (29). Using FACS, the authors observed that the clonogenic cells expressed similar surface antigens (see below for explanation of surface antigens) to marrow MSCs. Moreover, the authors observed that the clonal cells were capable of differentiating into adipocytes, osteoblasts, and, chondrocytes, demonstrating that adipose tissue contains a population of MSCs.

In order to determine if adipose is a comparable source of MSCs to bone marrow, recent investigations have compared yields and differentiation capacities of cells isolated from each tissue. For example, Ugarte et al. 2003 found no significant difference in the number of culture adherent cells per gram of stromal cells obtained from human marrow or adipose tissue (30). Yet more than double the average mass of adipose tissue (17g) could easily be isolated from each patient compared to bone marrow (7g). The authors also cultured isolated cells in various differentiation media. Ugarte et al. found no difference in the number of cells that developed lipid droplets (adipogenic cells), or the alkaline phosphate activity of osteogenic cells. However, when induced to differentiate into cartilage, adipose derived cells stained positive for chondrogenesis while marrow derived cells did not. Using similar methods, several other investigations have compared the ability of marrow and adipose cells to differentiate along these lineages and have demonstrated that cells from either tissue possess an equal capacity to become adipose, bone, and cartilage (31, 32). Taken together, the above evidence demonstrates that compared to marrow, a high number of MSCs capable of multi-lineage differentiation can be obtained from adipose.

1.4 Heterogeneity of adipose stromal cells

The stroma of adipose is composed of several different cell types, of which MSCs are the minority (>1%) (33). The main cellular component of adipose is mature adipocytes. Other major cell types include endothelial cells which comprise blood vessels, fibroblasts that produce extracellular matrix, and cells of hematopoietic origin including granulocytes, monocytes, and lymphocytes (34). During the isolation of MSCs from adipose stroma a heterogeneous population of cells will be obtained (mature adipocytes are easily removable as a supernatant due to their fat content and therefore low sedimentation coefficient). Because of this plurality of cell types, the cell population established from adipose tissue may not display a robust differentiation response when analyzed using histochemical techniques, compared to a culture with a higher proportion of stem cells. With this in mind, various investigators have exploited the expression of specific cell surface antigens (markers) to distinguish and separate MSCs from non-MSC stromal cells (33). According to the International Federation for Adipose Therapeutics and Sciences (IFATS) adipose-derived MSCs should express CD34, CD13, CD73, CD90, and CD105 and be negative for CD45, CD235a, and CD31 (33). In practice, however, the surface antigen profile attributed to adipose derived-MSCs is highly variable between research groups (see Table 1). In contrast, many non-MSC stromal cells express unique surface antigens that MSCs lack, and these can also be used for cell identification and separation. Described below are markers expressed by endothelial cell, fibroblasts, and cells of hematopeoitc origin, which together comprise the majority of adipose stromal cells.

Table 1.1 : Adipose derived-MSC marker profiles

Markers profile	Reference
CD 29, CD 44, CD 45, CD 90, MHC I, MHC II	35
CD 29, CD 44, CD 45	36
CD 29, CD 45, CD 54, CD 90, CD 106	37
CD 34, CD 44, CD 45, CD 90	38
CD 34, CD 45, CD 29, CD 44, CD 90	39
CD 105, CD 29, CD 45, CD 34	40
CD 11b, CD 45, CD 90	41
CD 34, Stro-1	42
CD 34, CD 44, CD 29, CD90	43
CD 29, CD 90, CD 54, CD 45, CD 11b, CD 106, MHC I, MHC II	44
CD 34, CD 45, CD 44, CD 90	45

CD54 (Intracellular adhesion molecule-1, ICAM-1) is a cell surface glycoprotein expressed on endothelial cells, fibroblasts, lymphocytes and monocytes, but absent on MSCs. CD54 belongs to the ICAM family of proteins. ICAMs possess roles in the development of the nervous system, in immune and inflammatory responses. ICAM-1 specifically participates in the trafficking of inflammatory cells, in adhesion of antigen-presenting cells to T-lymphocytes during antigen presentation, and in microbial pathogenesis (46). Stromal CD54 expression varies among studies (47). Blasi *et al.* found that 19.6% +/- 9.4 of stromal cells express CD54 (48). Similarly, Zhao et al. found that 11.0% +/- 0.7 of adipose tissue cells express CD54 (49). Conversely, other groups have reported that adipose stromal cell populations contain ~40% to more than 80% CD54

positive cells (50, 51). Recently, Zhao et al. proposed that CD54 may be used as a marker to distinguish adipose MSCs from local fibroblasts (49). Using magnet activated cell sorting, Zhou et al. separated CD54 positive cells from stromal cells. The authors found that the adipogenic and osteogenic differentiation capacity of the CD54 negative population was enhanced compared to the cultures containing CD54 positive cells.

CD45 (also called common leukocyte antigen, CLA) is a cell surface protein tyrosine phosphatase that controls lymphocyte development, signals emanating from integrin and cytokine receptors, and is expressed by all hematopoietic lineage cells (T lymphocytes, B lymphocytes, monocytes, granulocytes etc.) except for erythrocytes and platelets (52). Importantly, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) do not express CD45. IFATS recommends using CD45 to distinguish MSCs from specialized immune cells (33). Adipose stromal cell populations from either rat or human tissue are negative or possess low expression of CD45 positive cells (53-58).

CD31 (Platelet endothelial cell adhesion molecule-1, PECAM-1) is a cell surface adhesion protein expressed on most leukocytes, platelets, and on endothelial cells. CD31 plays an important role in regulating the attachment of leukocytes to endothelial cells on vessel walls and the migration of leukocytes through the vessel wall into tissues. In regards to endothelial cells, concentrated stores of CD31 have been observed in surface vascular membrane invaginations at endothelial cell junctions (59). ISCT/IFATS recommends the use of CD31 for negative selection against endothelial cells in adipose SVF populations (33). Analyses of human adipose SVF cell populations show that CD31

positive cells are present in adipose SVF. For example Gimble et al. 2006 found that ~20% of human adipose SVF express CD31 (60).

Taken together, the above evidence suggests that adipose stromal culture populations may be enriched for MSCs through the selective removal of cells expressing CD 54, CD45, and CD31. This strategy was therefore implemented to increase the proportion of MSCs in cell cultures established from rat adipose tissue (see below).

1.5 MSC Differentiation

In addition to their surface antigen profile, MSCs are defined by the ability to undergo adipogenic, osteogenic, and chondrogenic lineage specific differentiation (33). Moreover, adipose MSCs can give rise to brite cells (7, 8). In order to differentiate into these distinct cell types, MSCs must express unique sets of genes which ultimately produce functional proteins whose activities support the specialized functions of the differentiated cell. To study the process of MSC differentiation, various investigators have developed panels of molecular inducers that stimulate MSCs to undergo lineage specific differentiation *in vitro*. With these concepts in mind, reviewed below is information regarding methods for differentiating MSCs into bone, cartilage, fat, and brite cells.

1.5.1 Osteogenesis

The extracellular matrix of bone tissue contains mineral salt commonly called osteoid, which is a crystalline complex of calcium and phosphate also known as hydroxyapatite [$Ca_{10}(PO_4)6(OH)_2$] (62). Inorganic hydroxyapatite crystals comprise ~69% of the material found in bone tissue. These crystals are found deposited along

collagen fibrils within the extracellular matrix (62). Collagen type I protein is the second most abundant constituent of bone, accounting for most of the remaining 31% (63). Non-collagen proteins such as proteoglycans, sialoproteins, gamma-carboxyglutamic acid-containing proteins, and 2HS-glycoproteins are minor components (62).

The process of bone formation through osteogenesis is facilitated by flat/rectangular support cells known as osteoblasts which are derived from MSCs of the bone marrow stroma. As osteoblasts differentiate they gain the ability to secrete the constituents of osteoid into the extracellular matrix. Subsequently, osteoblasts become trapped in the bone matrix and give rise to osteocytes, which gradually stop secreting matrix (62).

The differentiation of MSCs into osteoblasts is governed by several transcription factors that control the expression of osteogenic specific genes, one of the most well characterized being Run2x. Run2x is expressed in MSCs early in skeletal development and throughout osteoblast differentiation (64). Overexpression of Run2x alone can induce osteogenesis *in vitro* and *in vivo*, increasing osteoid production and accelerating healing of bone defects, respectively (65). Takahashi et al. 2011 demonstrated that the overexpression of Run2x in the pre-adipocyte cell line, 3T3-E1, caused an increase in osteogenic genes (67). Conversely, Run2x-null mice display a complete absence of osteogenesis due to a differentiation arrest of osteoblasts (66).

In vitro differentiation of MSCs into osteoid secreting osteoblasts can be achieved through the use of a multi-week treatment strategy with dexamethasone, β-glycerophosphate, and ascorbic acid (68). Dexamethasone is a synthetic corticosteroid

not found naturally *in vivo* that imitates the actions of glucocorticoids (69). As early as 1985, Tenebaum and Heersche demonstrated that dexamethasone is capable of inducing osteogenesis in chick periosteum cells *in vivo* (70). Since this time, investigations have revealed that dexamethasone interacts simultaneously with several signalling cascades inducing Run2x transcription. Hamidouche et al. 2008 demonstrated that the FHL2 (four and a half LIM domains 2) protein mediates dexamethasone induced osteogenesis by activating the WNT/ β-catenin signaling which in turn activates the transcription of Run2x (71). Additionally, Phillips et al. 2006 observed that dexamethasone increase mitogen-activated kinase (MAPK) phosphatase (MKP-1) which in turn facilitates the dephosphorylation of a serine residue leading to enhanced Run2x activity (72).

In addition to dexamethasone, ascorbic acid and β-glycerophosphate are required for *in vitro* osteogenesis. Ascorbic acid stimulates osteogenesis by aiding in the production of collagen type I. In order to do this, ascorbic acid acts as a co-factor for enzymes that hydroxylate proline residues of the collagen precursor, pro-collagen. In the absence of ascorbic acid, proline cannot be hydroxylated and collagen chains are not able to form a proper helical structure (73). In addition to being an essential structural component of osteoid it is further hypothesized that the presence of collagen facilitates enhanced Run2x expression through the activation of cell surface intergrin receptors (68). Furthermore, β-glycerophosphate plays an important role in osteogenesis, however the mechanism through which β-glycerophosphate acts is unclear. It is believed that β-glycerophosphate acts as a source of organic phosphates that aid in extracellular matrix mineralization. Moreover β-glycerophosphate may also aid in the ability of alkaline phosphatase to hydrolyze organic phosphate into inorganic phosphate. This free

phosphate may provide the chemical potential for promoting mineral deposition on the surface of tissue culture plastic by osteoblasts (69).

1.5.2 Chondrogenesis

Cartilaginous tissue is composed of a dense extracellular matrix with a sparse population of specialized cells known as chondrocytes (74). The extracellular matrix is the principle functional component of cartilage and mainly comprises water, collagen, and proteoglycans. Water is the most abundant component of cartilage, contributing up to ~80% of its wet weight. Collagen is the most abundant macromolecule in the extracellular matrix, and makes up ~60% of the dry weight of cartilage. Collagen proteins contain a region consisting of 3 polypeptide chains wound into a triple helix. This structure provides cartilage with important shear and tensile properties, which help to stabilize the matrix. Type II collagen represents 90%-95% of the collagen in the matrix. It forms fibrils and fibers that intertwine aggregates of proteoglycans. Collagen types I, IV, V, VI, IX, and XI are also present, but in lesser amounts. Proteoglycans represent the second most abundant group of macromolecules in the extracellular matrix and account for ~10 to 15% of the wet weight. Proteoglycans are heavily glycosylated protein monomers consisting of a protein core with one or more linear glycosaminoglycan chains covalently attached. Cartilage contains a variety of proteoglycans including aggrecan, decorin, biglycan, and fibromodulin. The interaction between proteoglycans and type II collagen aids in providing compressive and tensile strength to the tissue (74).

Chondrocytes are the resident cell type in cartilage and are responsible for the production of the extracellular matrix during development through a process known as

chondrogenesis. At the onset of development of the skeletal system, mesenchymal stem cells commit to chondrogenesis and differentiate into pre-chondrocytes and then chondroblasts. These cells form the rudimentary basis for skeletal structures. In the center of future long bones, the cells become tightly packed and undergo stages of proliferation, hypertrophy, maturation into chondrocytes, and then apoptosis. Subsequently, bone-forming cells invade the lacunae of apoptotic chondrocytes and establish ossification centers, replacing the areas formerly occupied by cartilage with bone. This process continues until the bones have elongated to a mature length (75). At the either end of developing long bones, articular joints form where mesenchymal stem cells located in presumptive joint areas differentiate into pre-chondrocytes and then undergo apoptosis to create joint cavities. Chondroblasts that line the cavities develop into cartilage producing chondrocytes, forming the cartilaginous lining of long bones (76).

In vivo, the commitment of MSCs to the chondrogenic lineage is initiated by the sonic hedgehog signaling pathway through the increased expression of bone morphogenetic proteins (BMPs), which direct MSCs towards chondrogenesis (76). Early chondrogenesis is also characterized by high expression levels of the transcription factor Sox9 which is a key transcription factor for chondrocyte development (77). For example, the inactivation of Sox9 in mouse embryos specifically in early limb bud MSCs prior to cartilage formation, results in a complete absence of cartilage in the appendages (78). Sox9 is initially responsible for repressing the expression of the osteogenic factor Run2x. Through repression of Run2x, Sox9 ensures chondrogenic commitment of MSCs over osteogenic development (79). Two other Sox family members, L-Sox5 and Sox6 are required for Sox9-mediated transformation of cells into chondroblasts (80, 81).

Moreover, Sox9 in concert with L-Sox5 and Sox6, maintain the chondrocyte phenotype in developed cartilage by activating the expression of several cartilage specific genes for collagen types II, IX, and XI and the proteoglycan, aggrecan (82, 83).

In order to induce chondrogenesis of MSCs *in vitro,* culture medium is supplemented with specific growth factors, hormones, and synthetic compounds. Transforming growth factor β (TGFβ) peptides are common supplements. These peptides are secreted as one of four isoforms and act as signaling molecules by binding heteromeric cell surface serine/threonine kinase receptor complexes consisting of dimeric type I and type II receptors. Binding of the ligand to a type II receptor activates the type I receptor which results in the phosphorylation of cytoplasmic Smad molecules. Subsequently, activated Smads translocate to the nucleus and act as transcriptional co-factors and directly influence gene transcription (84). TGFβ is included in MSC chondrogenic mediums in order to induce the secretion of cartilaginous ECM constituents. For example, Danisovic et al 2007 demonstrated that TGFβ I treatment leads to increased collagen type II and aggrecan gene expression (85). Similarly, Thrope et al 2010 showed that TGFβ 3 increases the level of glycosaminoglycan synthesis of MSCs (86).

Other common constituents of chondrogenic medium include insulin, selenium, transferrin, ascorbic acid and dexamethasone. Insulin is a peptide hormone produced by β cells of the pancreas in order to modulate glucose metabolism. Systemic insulin binds to cell surface insulin receptors and induces the translocation of glucose transporters to the plasma membrane in order to increase cellular glucose influx (87). Mueller et al. 2013 demonstrated that insulin is required for human MSC chondrogenic differentiation and

showed that a positive correlation exists between the insulin dosage and the synthesis glycosaminoglycan and collagen of MSCs (88). Transferrin is an essential protein that is known to transport iron into cells, where iron aides in facilitating the hydroxylation of proline required for collagen synthesis and selenium is an essential trace element that increases the activity of glutathione peroxidases in protecting cells from oxidative stress (89-91). Additionally, ascorbate is a cofactor required in the production of collagen type II synthesis in chondrocytes and has been observed to influence glycosaminoglycan synthesis (92, 93). Dexamethasone is a synthetic glucocorticoid and is also commonly included in chondrogenic media in order to increase collagen type II synthesis by increasing levels of Sox9 in MSCs (94). In addition to media supplements, MSCs may be formed into a cell pellet by centrifugation prior to differentiation. It is postulated that the induced mechanical force and limited oxygen supply that accompany this cell formation aid in the commitment of MSCs to the chondrogenic lineage (95).

1.5.3 Adipogenesis (white adipose)

The mature adipocyte contains a single large fat droplet surrounded by a thin rim of cytoplasm that lies between the droplet and the plasma membrane. When triggered by lipolytic hormones, fatty acids are derived from triglycerides within the fat droplet by cytoplasmic hormone-sensitive lipase and adipocyte-triglyceride lipase that translocate to the surface of the droplet. The fatty acids are then released into the blood stream to supply peripheral tissues. During periods of excess caloric intake, lipogenic enzymes localize in the cytoplasm and endoplasmic reticulum, synthesize triglyceride, which is incorporated into the fat droplet (102).

At approximately the beginning of the second third of gestation, large and small arterioles can be detected in the vicinity of small developing fat cell clusters at various sites (buccal, neck, shoulder, perirenal). Adipose appears and develops in these areas where it remains after birth. Primitive fat cell clusters are then formed, composed of densely packed fat cells adjacent to capillaries. The last stage of growth of adipose is mainly due to an increase in size of fat cell clusters surrounded by mesenchymal stroma, which condenses rapidly and then thickens to form septa among the clusters (103). The development of MSCs into terminally differentiated adipocytes occurs as discrete phases. The first being the commitment phase in which the stem cell is converted into a preadipocyte. The preadipocyte cannot be distinguished morphologically from its precursor but has lost the potential to differentiate into other cell types. The events governing the commitment phase have not been well characterized, largely because of the predominant use of preadipocyte cell lines to study adipogenesis. Following commitment to the adipogenic lineage, preadipocytes will experience period of growth arrest at the the G_1/G_o cell cycle boundary. Preadipocytes receiving appropriate mitogenic and adipogenic signals will then synchronously re-enter the cell cycle and undergo several rounds of cell division referred to as mitotic clonal expansion. As cells complete clonal expansion, terminal differentiation occurs which is characterized by expression of the enzymatic machinery required for lipogenesis and triacyl glycerol synthesis which is responsible for lipid accumulation within the differentiated adipocyte (104).

The central regulator of adipogenesis is the transcription factor peroxisome proliferator-activated receptor γ (PPARγ). When activated, PPARγ interacts with the retinoid x receptor, forming a heterodimer which binds DNA at peroxisome proliferator

response elements in the promoter of target genes. The binding of the heterodimer is required for the activation many adipocyte specific genes involved lipid breakdown and storage (105). The importance of PPARγ in adipogenesis has been demonstrated in several ways. For example, the overexpression of PPARγ in non-adipogenic fibroblasts produces cells with an adipocyte-like phenotype (106). Moreover, Rosen et al. 1999 created chimeric mice derived from both wild-type embryonic stem cells and cells with a homozygous depletion of PPARγ. This strategy allows for the measurement of the contribution of PPARγ null cells to others tissues in healthy animals (107). The researchers observed that null cells were excluded from WAT but not several other tissues. Moreover, PPARγ heterozygotes animals exhibit resistance to diet-induced obesity (108).

In order to induce adipogenesis of MSCs *in vitro,* culture medium is supplemented with specific hormones such as insulin and synthetic compounds including 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and rosglitazone. Insulin is a polypeptide hormone that binds to cell surface insulin receptors (IR) in order to initiate a number of downstream signaling cascades (87). The presence of insulin appears to be required for *in vitro* adipogenic differentiation. However, the mechanism through insulin induces adipogenesis is not fully understood. The loss of insulin-receptor substrate (IRS), a signaling protein activated by IR, inhibits adipogenesis (109). Moreover, the specific inhibition of phosphatidylinositol 3-kinase (an effector protein activated by IRS) also blocks adipogenesis (110). Rosglitazone is a potent PPARγ agonist. Rosiglitazone treatment increases the adipogenic response *in vitro* and increases the number of adipocytes in dogs and rats (111). Through the activation of PPARγ, rosiglitazone

initiates the expression of a number of adipogenic genes (105). Furthermore, IMBX is a phosphodiesterase inhibitor increases intracellular cyclic adenosine monophosphate (cAMP) levels. cAMP subsequently activates the cAMP response element-binding protein (CREB) which increases PPARγ expression (105). Lastly, dexamethasone is a synthetic glucocorticoid which activates glucocorticoid receptors. The adipogenic differentiation of MSCs is accelerated by dexamethasone increases the expression of adipogenic transcription factors PPARγ and C/EBPα (CCAAT/enhancer binding protein α) (105).

1.5.4 Adipogenesis (Brown adipose and Brite cells)

Brown adipose tissue (BAT) tissue develops during embryogenesis within intrascapular regions and peripheral tissues and is the key site of heat production in small mammals and infant humans. The heat produced by brown adipocytes is a result of the non-shivering thermogenesis pathway (112). Deficits in body temperature are recognized by the central nervous system and are relayed via sympathetic neurons which releases norepinephrine (NE) at BAT depots. Subsequently, NE binds to surface β_3-adrenergic receptors which initiate the breakdown of stored triglycerides into fatty acids that activate UCP1. These fatty acids can also be oxidized by mitochondria. However, this potential energy of the proton motive force established by respiration is in part dissipated by protons transporting through UCP1 instead of ATP-synthase, resulting in increased oxygen consumption and substrate oxidation. Ultimately, lipid oxidation by brown adipocytes releases energy in the form of heat which is transferred throughout the organism via the circulatory system, thus warming the organism (112).

Compared to infants and small mammals, the amount of BAT within adult humans is relatively small (12). Interestingly, in addition to BAT cells, populations of inducible brown adipocyte-like cells, known as brite cells have also been found within WAT. Brite cells arise in WAT from resident populations of stem cells. Morphologically, brite cells resemble brown adipocytes, possessing lipid droplets and a high mitochondrial content. Moreover, brite cells express UCP1 and the signal transduction pathway that regulates non-shivering thermogenesis in response to NE (113, 114). In order to induce the differentiation of WAT derived stromal vascular fraction (SVF) stem cells into brite cells *in vitro*, inducers of UCP1 expression have been utilized. The promoter region of the *ucp1* gene contains cyclic AMP (cAMP) response elements (CRE) in addition to a peroxisome proliferator activated receptor (PPAR) response element (PPRE) and a thyroid hormone response element (TRE). In the presence of NE, brown adipocytes experience an increase in the concentration of phosphorylated cAMP binding protein (pCREB) (112). Rim & Kozak 2002 reported that pCREB binds to CRE in the ucp1 promoter region and activates its transcription (115). Similarly, synthetic agonists of the transcription factor peroxisome proliferator receptor γ (PPARγ) such as rosiglitazone and indomethacin activate PPARγ, causing it to bind to PPRE in the promoter region of ucp1 and increase transcription (112). Furthermore, in the presence of triiodothyronine (T_3), thyroid receptors translocate to the nucleus and bind TRE and in turn enhance ucp1 expression (112). Recent investigations have reported that incubating WAT derived SVF cells with T_3 combined with PPARγ and/or NE is sufficient to induce the differentiation into brite cells capable of initiating a thermogenic response to NE (116-120).

In addition to specific stimulators of UCP1, IBMX in combination with insulin and dexamethasone is commonly utilized to induce brite cell and brown adipocyte differentiation (116-121). IBMX increases cAMP levels through the inhibition of degradation cAMP phosphodiesterases (122), thus increasing CRE activity. Insulin and dexamethasone (a synthetic glucocorticoid) interact with IR and glucocorticoid receptors (GC receptors), respectively (123). Little is known concerning the specific molecular mechanisms of these molecules in the differentiation process. However, the presence of glucocorticoid alone induces differentiation of brown adipocytes *in vitro* (124). Moreover, the presence of insulin and the activation of its downstream effector, IRS, is necessary for brown adipogenesis (125).

1.6 Therapeutic use and potential of MSCs

The ability of MSCs to differentiate into osteoblasts, chondroblasts, and brite cells has attracted much interest for their use in combating age-related pathologies. Moreover, MSCs are ideal for regenerative therapies due to the fact that they do not induce adverse immune responses in autologous (self) or allogeneic (separate individual) transplants. Firstly, the osteogenic potential of MSCs has benefit in an orthopedic setting (126). Osteoporosis is a condition of older individuals and is characterized by skeletal fragility due to progressive bone loss. Bone becomes sufficiently weak that fractures occur with minimal trauma (127). The use of MSCs for fracture repair has been tried successfully in animal models. Autologous bone marrow-derived MSCs have been expanded in culture, loaded onto ceramic cylinders, and implanted into 8-mm segmental defects in rat femurs with successful bone formation 8 weeks later (128). The same group then successfully demonstrated bone formation in segmental defects in adult rats by human marrow-

derived MSCs (129). Several other animal studies using implanted autologous MSCs have also resulted in successful bone regeneration (130, 131). Thus, the injection MSCs into bone defects is a promising therapy to treat fractures caused by age-related osteoporosis.

In addition to bone repair, MSCs also have applications in regenerating cartilage tissue. Osteoarthritis is a disease with a high prevalence among elderly adults. Osteoarthritis is characterized by degeneration of the cartilaginous lining of joints of long bones causing pain and limited mobility (132). MSC transplantation has been shown to be effective for cartilage repair in animal models and human patients. For example, Murphy et al. 2003 intra-articularly injected goats in which osteoarthritis was induced by surgery (133). Labelled MSCs were not found in large numbers in the cartilage area. However, regeneration of the tissue was clearly evident in animals receiving cells in comparison to controls. Similarly, undifferentiated and pre-differentiated MSCs on scaffolds yielded cartilage repair in rabbits and sheep models of osteoarthritis. Human trails demonstrated beneficial effects of MSCs for osteoarthritic patients. For example, Orozco et al. 2013 identified radiological evidence of osteoarthritis in 12 patients (134). Individuals were then treated with autologously expanded marrow MSCs by intra-arctcilar injection. Following 1 year 11 out of 12 patients displayed improved cartilage quality in the treated areas. Taken together, MSCs based therapy is a promising strategy to ameliorate the symptoms of age-associated osteoarthritis.

Sedentary living and the consumption of calorie-dense food has led to a rise in obesity throughout the developed world. This is especially true in the elderly, among whom obesity-associated pathologies such as hyper-tension, type 2 diabetes,

hyperglycemia, are highly prevalent (135, 136). Obesity-related conditions often arise due to a misbalance in energy intake relative to expenditure, resulting in excess energy stored as lipid in WAT (135). Thus, increasing non-shivering thermogenic activity is a potential strategy to combat obesity. Investigations into the relationship between non-shivering thermogenesis and lipid metabolism have revealed that enhanced BAT activity is capable of conferring anti-obesity properties. For example, Feldmann et al. 2009 developed UCP1-defiecient mice and demonstrated that transgenic mice gained more weight than wild-type animals (137). Similarly, overexpression of UCP1increases thermogenesis and prevents weight gain (138, 139). Moreover, mice with increased BAT activity display improvements in conditions associated obesity such as increased insulin sensitivity and glucose tolerance (140, 141). Considering that resident MSCs in WAT depots possess the ability to differentiate into BAT-like cells, increasing thermogenic activity by inducing MSC differentiation into brite cells is a potential strategy to combat obesity.

The transplantation of cells between individuals of the same species (allogeneic transplantation) not receiving immune suppressive therapy typically results in rejection due to cell death caused by activation of host immune systems. An accumulating body of evidence suggests that allogeneic MSCs suppress host immune cells allowing them to avoid rejection (142). Following tissue implantation, host lymphocytes become activated as part of the innate immune response. Activated lymphocytes subsequently proliferate and then differentiate into effector cells that infiltrate foreign tissues causing apoptosis within the transplant (143). In 2002, Bartholomew *et al.* showed that the addition of primary MSCs to cultures of pre-activated allogeneic lymphocytes suppressed

lymphocyte proliferation (144). In order to test the immune suppressive potential of MSCs *in vivo*, allogeneic skin grafts were performed on baboons following injection of donor matched MSCs. The authors observed that MSCs extended graft survival from 7 days (no MSC control) to 11 days. Furthermore, Liechty *et al*. 2000 reported that human MSCs could persist as long as 13 months after injection into pre-immune (immune system has not developed) and immune competent fetal sheep (145). In contrast human or allogeneic hematopoietic stem cells did not persist in this model. Taken together, the above evidence suggests that allogeneic MSCs do not provoke adverse immune responses. Considering this, MSCs may potentially be isolated and stored either for autologous (self-transplant) or allogeneic transplantation.

1.7 Project Objectives

MSCs isolated from adipose tissue are an ideal source of stem cells because they are easily accessible and possess the ability to differentiate into specialized cell types. However, several unique challenges currently limit the usefulness of adipose as a source of MSCs for regenerative medicine. The majority of cells within adipose stroma are non-MSC cell types such as fibroblasts, endothelial cells, and hematopoietic cells. Because MSCs represent a small fraction of total stromal cells, adipose stromal suspensions diluted with non-MSC cells may possess a limited differentiation capacity. Furthermore, MSCs from adipose have traditionally been isolated using collagenase in order to liberate stromal cells from the surrounding extra-cellular matrix. This method is not ideal because the sample will be contaminated with potentially toxic exogenous enzyme that cannot enter a patient. Moreover, recruitable populations of brite cells are believed to reside within WAT depots. Recent evidence suggests that inducers of UCP1 expression can

stimulate resident WAT MSCs to differentiate into brite cells. With the above issues in mind, the overall purpose of this thesis is to further develop novel methods for isolating and differentiating adipose-derived MSCs into desired cell types.

This Thesis addresses the following questions:

1. Does the removal of non-MSC cell (fibroblast, endothelial, hematopoietic) cell types from a heterogenous population of adipose stromal cells increase the proportion of stem cells capable of differentiation *in vitro*?
2. Can MSCs be isolated from Human adipose using a non-enzymatic method with an efficiency comparable to the collagenase based method?
3. Can differentiation medium containing known stimulators of UCP1 expression induce the differentiation of WAT stromal cells into thermogenically competent brite cells?

Chapter 2: Development of isolation, enrichment, and differentiation protocols for adipose derived stem cells using rat adipose tissue

Objectives: Assess effectiveness of standard protocols for the isolation, adipogenic differentiation, and ostegenic differentiation on stromal vascular cells isolated from rat adipose. Furthermore, to determine if removal of non-mesenchymal stem cell (fibroblast, endothelial, hematopoietic) cell types from a heterogenous population of rat adipose stromal cells will increase the proportion of stem cells capable of differentiation *in vitro*.

Hypothesis: MSCs will be isolated from rat adipose. Depletion of non-mesenchymal stem cells from the heterogeneous population of stromal vascular cells will give rise to an enriched MSC culture capable of differentiation into osteoblasts and adipocytes.

Abstract

The majority of cells within tissue stroma are non-MSC cell types such as fibroblasts, endothelial cells, and hematopoietic cells. MSCs represent a small fraction of total stromal cells. Thus, cultures diluted with non-MSC cells may possess a limited differentiation capacity. Adherent, proliferative stromal cultures were established from rat adipose and bone marrow. Cultures were subsequently exposed to medium containing inducers of adipogenic or osteogenic differentiation. Following the differentiation treatment period, oil red o and alizarin red staining for adipose and bone differentiation, respectively, was performed. Staining revealed that minimal adipogenic differentiation was achieved while osteogenic differentiation was absent in stromal cultures. Separate groups of stromal cells were then incubated with anti-CD 54, 45, and 31 antibody-coated magnetic beads and subjected to magnetic sorting in order to remove non-MSC stromal cells. Cell counting following sorting showed that the separation process removed bead bound cells from cell suspensions. However, neither sorted nor non-sorted stromal cells experienced a strong differentiation response. These results suggest that the removal of non-MSC stromal cells had no effect on the osteogenic or adipogenic capacity of stromal cultures. Moreover, a lengthy culture period was utilized prior to sorting. It is likely this extended culture period affected the differentiation capacity of the stromal cells. Thus, future work should assess separation procedures that minimize the time cells spend *in vitro* prior to differentiation.

2.1 Introduction

Mesenchymal stem cells (MSCs) comprise a minute percentage of total cells (>1%) present within adipose tissue. The majority of cells within the tissue stroma are cells of hematopoietic origin such as erythrocytes and leukocytes or connective tissue cells such as adipocytes, fibroblasts and endothelial cells (33). Therefore, during any protocol for isolation of MSCs from adipose tissue stroma a heterogeneous population of cells will initially be obtained. Because of this plurality of cell types, the cell population established from adipose tissue may not display a robust differentiation response when analyzed using histochemical techniques, compared to a culture with a higher proportion of stem cells. Moreover, it can be difficult to obtain a large number of adipose derived cells and these may be significantly diluted by non-MSCs, thus it is necessary to develop methods to maximize both the yield and 'purity' of MSCs from adipose tissue. Since it was anticipated that access to human adipose tissue samples would be extremely limited, it was necessary to develop the methodology for isolating and enriching stromal vascular derived MSCs using a more readily available source of adipose tissue. The laboratory rat *Rattus norvegicus* was chosen because of its convenience as an experimental model, availability, and the size of adipose stores that could be isolated. Therefore, in this chapter, rats were used to establish the collagenase-based digestion protocol for isolating MSCs from the adipose stromal vascular fraction, to establish and validate protocols for differentiating (and for evaluating differentiation) MSCs into adipocytes, osteocytes, and to test an immuno-magnetic negative selection method for enriching MSCs.

With respect to the enrichment of MSCs, according to IFATS adipose-derived MSCs should express CD34, CD13, CD73, CD90, CD105 and be negative for CD45,

CD235a, CD31 (33). However, the surface antigen profile attributed to adipose derived-MSCs is highly variable between research groups (table 1.1). In contrast, many non-MSC stromal cells express unique surface antigens that can be used for cell sorting and selection. For example endothelial cells express CD31, fibroblasts express CD54, and cells hematopoietic cells express CD45 while MSCs do not (33, 59, 49, 147). With these issues in mind, a negative selection strategy was explored to enrich the MSC by removing these other cell types.

2.2 Materials and Methods

2.2.1 Materials

3-isobutyl-1-methylxanthine (IBMX), ascorbate-2-phosphate seqimagnesium salt hydrate, biotin, β-glycerophosphate disodium salt, bovine serum albumin (BSA) fraction V, cetylpyridium chloride, dexamethasone, d- pantothenate, dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), Hank's balanced salt solution, low glucose Dulbecco's modified eagles media (DMEM) powdered media, human recombinant insulin, paraformaldehyde, penicillin/ streptomycin, rosiglitazone, and 0.25% trypsin-EDTA were purchased from Sigma Aldrich.

Calcium chloride, glutamine, HEPES, potassium chloride, potassium diphosphate, monobasic potassium phosphate, sodium bicarbonate, sodium chloride, sodium hydroxide and dibasic sodium phosphate were purchased from Fischer Scientific.

DMEM/ F-12 powdered media with L-glutamine, and Ham's F-12 powdered media with L-glutamine were purchased from Invitrogen. Collagenase type I was purchased from Worthington Biochemical. Hydrochloric acid, isopropanol, and sodium hydroxide were purchased from Caledon. Alizarin red and oil red O were purchased from Santa Cruz Biotechnology. Biotinylated anti-rat CD54 and CD45 were purchased from Biolegend. Biotinylated anti-rat CD31 was purchased from BD biosciences. M-280 streptavidin coated Dynabeads were purchased from Invitrogen. Sodium pentobarbital was purchase from Bimeda-MTC animal health Inc. Isoflurane was purchased from Pharmaceutical Partners of Canada.

3T3-L1 cell line was purchased from Eton Biosciences. Saos-2 cell line was a gift from the Dr. Ward lab (Brock University, St. Catharines, Ontario, Canada).

2.2.2 Animals and primary cell line isolation

The use of experimental animals was approved by the Brock University ACUC. Animal euthanization: young adult Long Evans rats were euthanized using either decapitation with a guillotine, pentobarbital sodium injection (2ml/ 4.5kg body weight) or inhalation of isoflurane plus heart excision using the Dispomed Moduflex Compact anesthesia machine. (table 1). *Adipose derived stromal vascular cells*: excised intra-peritoneal adipose tissue was subjected to collagenase digestion. Tissue was minced and washed multiple times with warm PBS until a clear infranatant solution was obtained. An equal volume of warm collagenase solution (0.1 % collagenase type I, 1% BSA in PBS) was added to the tissue and shaken at 75 RPM for 60 min using the Laboratory Water Bath Shaker Model G76 (New Brunswick Scientific). Digested tissue was vortexed for 15 seconds and subsequently centrifuged at 350 *x g* for 5 minutes using the Sorvall RC 5C Plus centrifuge. Samples were then vortexed again for 10 seconds. Samples were again centrifuged for 5 minutes at 350 *x g*. The supernatant of oil and collagenase solution was subsequently removed. Cells were then re-suspended in PBS and centrifuged at 350 *x g* for 5 minutes. The supernatant was then removed and cells were re-suspended in growth media (DMEM/ F-12, supplemented with 2.438 g/L sodium bicarbonate, 10% FBS, 1% penicillin/streptomycin). Cells were seeded on either 60mm or 100mm tissue culture treated dishes (Sarstedt) at ~0.16 ml tissue/cm^2 (2).

Bone marrow derived stromal vascular cells: Femurs from euthanized rats were excised and removed of muscle and connective tissue. Femurs were subsequently placed in cold Hank's balanced salt solution (HBSS) for 5 min. Using bone cutting scissors, the ends of femurs were removed. Subsequently, the marrow plug was flushed from the bone

using a 27 gage needle filled with cold HBSS. The marrow plug was re-suspended into a cell suspension and centrifuged at 350 *x* g for 10 min. The cell pellet then was re-suspended in growth media (Low glucose DMEM supplemented with 10% FBS, 5.96 g/L HEPES, 1% penicillin/streptomycin). Cells were seeded on tissue culture treated dishes (Sarstedt) at a density of 1.94 x 10^6 cells/cm^2 (17).

2.2.3 Cell Culture:

Stromal vascular cells obtained from either adipose or bone marrow were cultured in growth media at 37˚C, 5% CO_2 and 3% O_2. Once passage 0 cultures reached ~70-80% confluence the cultures were split at a ratio of 1:2 using 0.25% trypsin-EDTA solution. Adipose derived cells were further subcultured for 1-2 more passages, allowed to reach confluency, placed on 60mm plates and exposed to osteogenic medium or media containing vehicle controls. Alternatively adipose derived or bone marrow derived cells were subcultured until passage 5 and subjected to magnetic separation. Following separation cells were plated on a 100 mm tissue culture treated dish and allowed to reach confluency (passage 6). Subsequently, cells were exposed to trypsin and plated on 35 mm tissue culture treated dishes (NEST) at a density of 15 000 cells/cm^2. Once reaching confluency cells were transferred to a 20% O_2 incubator and were treated with differentiation treatments or corresponding vehicles.

Osteogenic Treatment: cells were cultured in growth media supplemented with 0.1 μM dexamethasone dissolved in DMSO, 0.01M β-glycerophosphate and ascorbate-2-phosphate dissolved in media for 21 days (18). Osteogenic control cells were cultured in growth media supplemented with an equivalent amount of DMSO (0.01%) until reaching

until 90-100% confluence (6 days). Osteogenic and control media was changed every 2 days.

Adipogenic Treatment: cells were cultured in adipogenic media consisting of: growth medium supplemented with 3% FBS, 1% pencilin/streptomycin, 200 nM human insulin dissolved in 4mM HCl, 66 μM biotin dissolved in 1M NaOH, 0.25 mM IBMX dissolved in DMSO, 34 uM pantothenate dissolved in water, 1 μM dexamethasone dissolved in ethanol, 5 mM and rosiglitazone dissolved in DMSO for 3 days. At day 4 of treatment, adipogenic cells received adipogenic maintenance media (adipogenic media without IMBX or rosiglitazone) for 8 days (19). Adipogenic control plates received growth medium supplemented with 3% FBS, 1% pencilin/streptomycin, 0.2% DMSO, 0.1% ethanol, 0.1% 1M NaOH, and 0.1% 4mM HCl until cells reached 90-100% confluence (6 days). Adipogenic and control media was changed every 3 days.

Osteosarcoma cells: Saos-2 human osteosarcoma cells were seeded on 35 mm tissue or 60mm culture treated plates at 10 000 cells/cm^2. Cells were cultured in Ham`s F-12 supplemented with 10% FBS, 1% penicillin/streptomycin, 28 mM HEPES, 1.1 mM $CaCl_2$, and 2.0 mM L-glutamine and incubated at 3% O_2, 5% CO_2. At 70-80% confluence, cells were moved to 20%, O_2, 5% CO_2 and cultured in osteogenic media consisting of Ham's F-12 supplemented with 50 μM ascorbate-2-phosphate, and 10 nM β-glycerophosphate dissolved in media and 0.1 μM dexamethasone dissolved in DMSO for 21 days. Control cells received growth media supplemented with an equivalent amount of DMSO (0.01%) for 6 days. Control and osteogenic media was changed every 2 days.

Preadipocytes: 3T3-L1 mouse preadipocyte cells were seeded on 35 mm tissue culture treated plates at 10 000 cells/cm^2. Cells were cultured in high glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin at 37˚C, 5% CO_2, 3% O_2. At 90-100% confluence, cells were moved to 20% O_2, 5% CO_2 and were cultured in adipogenic media consisting of: DMEM/ F-12 supplemented with 3% FBS, 1% penicillin/ streptomycin, 200 nM human insulin dissolved in 4mM HCl, 66 μM biotin dissolved in 1M NaOH, 0.25 mM IBMX dissolved in DMSO, 34 uM pantothenate dissolved in water, 1 μM dexamethasone dissolved in ethanol, 5 mM and rosiglitazone dissolved in DMSO for 3 days. At day 4 of treatment, adipogenic cells were received adipogenic maintenance media (adipogenic media without IMBX or rosiglitazone) for 8 days. control plates received DMEM/F-12 supplemented with 3% FBS, 1% penicillin/ streptomycin, 0.2% DMSO, 0.1% ethanol, 0.1% 1M NaOH, and 0.1% 4mM HCl until cells reached 90-100% confluence (6 days). Control and adipogenic media was changed every 3 days.

2.2.4 Magnetic Separation

Magnetic separation was conducted according to manufacturer's instructions. The following process was performed in a series of 3 isolation events conducted in succession, each with a different antibody. The first isolation used anti- CD 31 antibodies, the second anti-CD 54 antibodies, and the third anti-CD 45 antibodies. 0.25 mg of streptavidin conjugated magnetic beads (Dynabeads) were washed by being placed in an eppendorf tube containing cold isolation buffer (PBS supplemented with 2mM EDTA and 0.1% FBS). Subsequently, beads were taken out of solution using the

DYNAL magnet (Invitrogen) for 1 min. The supernatant was removed and the beads were resuspended in fresh isolation buffer. 2.5ug of antibody was placed into the tube containing pre-washed beads suspended in isolation buffer. Beads were incubated with antibody for 30 min with gentle rocking. Antibody conjugated beads were washed with isolation buffer using the DYNAL magnet and then incubated with 1 x 10^7 passage 5 cells derived from either adipose or bone marrow. Cells and antibody conjugated beads were incubated at 4^{o}C with gentle rocking for 30min. Bead-bound cells were separated from unbound cells using the Dynamag 15 magnet (Life technologies) for 2 min. The superanatant containing unbound cells was transferred to a fresh tube. Unbound cells were then counted using a hemocytometer. The unbound cells were subsequently centrifuged, resuspended in growth media and seeded on a 100 mm tissue culture treated plate.

2.2.5 Staining

Alizarin red: Osteogenic differentiation was detected using alizain red staining. Cells were prepared for staining by fixation in 4% paraformaldehyde (pH 7) solution at 4˚C overnight. Fixed cells were incubated in 40 mM alizarin red solution (pH 4.2) for 20 min. Following staining, excess dye was removed with distilled water (20). *Oil Red O*: adipogenic differentiation was detected using oil red O staining. Cells were prepared for staining by fixation in 4% paraformaldehyde (pH 7) solution at 4˚C overnight. Oil red O was prepared as 0.35% stock in 99% isopropanol. At time of use a 60% working solution was made by diluting stock solution in distilled water. The working solution was mixed and allowed to sit for 30 min and filtered before use. Fixed cells were incubated in 60% working solution for 1 hour. Excess dye was removed with distilled water (21).

2.2.6 Dye elution

Osteogenic differentiation was assessed by measuring the absorbance of alizarin red eluted from stained cells. Alizarin red was eluted by placing cells in 10% cetylpyridinium chloride (CPC) dissolved in 10mM sodium phosphate for 1 hour (7). Adipogenic differentiation was assessed by measuring the absorbance of oil red o eluted from adipogenic and control plates. Oil red o was eluted by placing stained cells in 99% isopropanol (elution of dye is immediate) (8). Alizarin red and oil red O elutant solutions were pipetted into 96 well flat optical bottom plates (Nalge Nunc International). The absorbance of the alizarin red elutant was read at 550nm and the absorbance of the oil red O elutant was read at 540 nm using the Bio-Tek Powerwave HT Microplate Spectrophotometer and KC4 software.

2.2.7 Imaging

Bright field and phase contrast images of cells in culture were captured using the Hund Wilovert S inverted microscope from Fischer Scientific. Bright field images of stained cells and were obtained using the Nikon eclipse 80i microscope.

2.2.8 Statistical analysis

Dye elution and ImageJ data were analyzed using the Student's T-Test with a one tailed distribution. Data were analyzed on GraphPad Prisim version 5. $\alpha = 0.05$ was used to determined statistical significance. In each analysis, technical replicates (individual cultures) were treated as biological replicates in order to achieve statistical significance.

Table 2.1: Method and purpose of separate animal euthanizations

Gender	Euthanization Method	Tissue harvested	Use of tissue (results section)
Female	Decapitation	Adipose	2.4.1
Female	Decapitation	Adipose	2.4.1
Female	Decapitation	Adipose	2.4.1
Female	Pentobarbital Sodium	Adipose	2.4.2
Female	Isoflurane + heart excision	Adipose	2.4.2
Female	Isoflurane + heart excision	Adipose, Bone marrow	2.4.2-2.4.3
Female	Isoflurane + heart excision	Bone marrow	2.4.3
Female	Isoflurane + heart excision	Bone marrow	2.4.3

2.3 Results

2.3.1 Preliminary isolation of rat adipose SVF cells

It was anticipated that human adipose tissue available for experimentation would be limited. Considering this, it was desirable to develop a method of isolating SVF cells using rat adipose prior to using human tissue. Adipose from three young adult rats was subjected to collagenase digestion and the stromal vascular cells obtained were subsequently placed in growth medium and seeded onto tissue culture dishes. Collagenase digestion yielded cultures containing proliferative cells which achieved approximately 80-90% confluence 2-4 days following seeding (Figure 2.1).

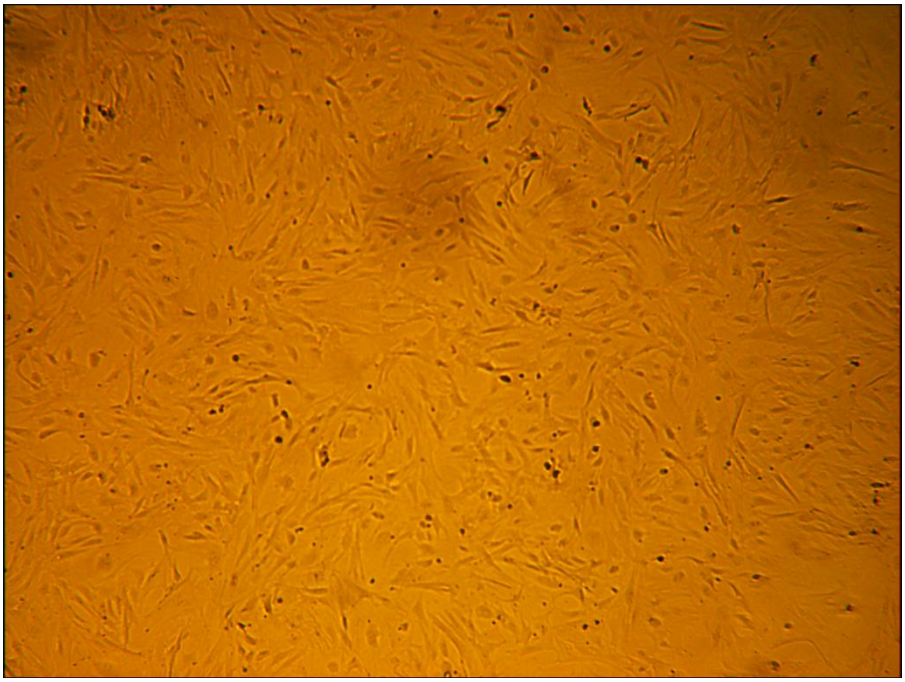
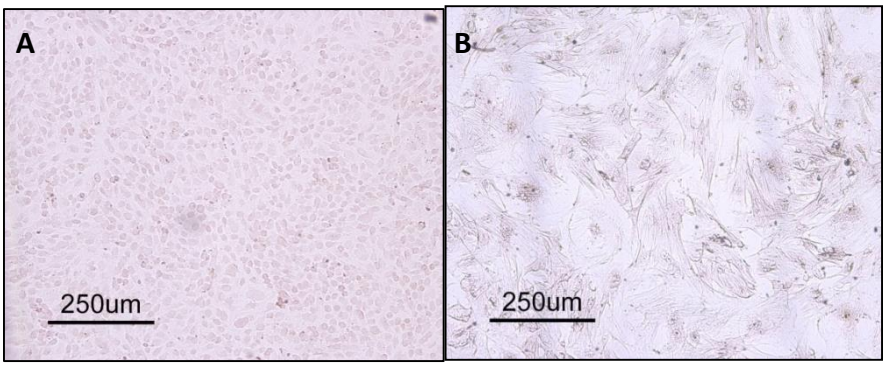


Figure 2.1. Rat adipose-derived stromal vascular cells in culture following collagenase digestion. Image at 4x magnification.

2.3.2 Osteogenic differentiation of adipose derived SVF cells

A central feature of MSCs *in vitro* is the ability to undergo osteogenic, adipogenic, and chondrogenic differentiation. The combination of dexamethasone, ascorbic acid, and β-glycerophosphate supplemented into growth medium has been previously demonstrated to induce osteogenic differentiation of MSCs *in vitro* (148). In order to determine if cells isolated from rat adipose were capable of osteogenesis, cells were cultured in the presence of osteogenic differentiation medium consisting of 50 μM ascorbate-2-phosphate, 10 nM β-glycerophosphate, and 0.1 μM dexamethasone for 21 days (n=9). Following the treatment period, cultures were fixed and stained with alizarin red to assess whether the cells produced a calcified matrix.

Saos-2 osteosarcoma cells are an immortal cell that have been previously demonstrated to undergo osteogenic differentiation and was used here as a positive control of osteogenic differentiation. Saos-2 cells (n=3) were exposed to the same treatment and staining conditions as adipose derived cells. Saos-2 cells cultured in differentiation medium displayed a high level of staining throughout the culture monolayer compared to those cultured in control medium (Figure 2.2 a, b). Compared to differentiated Saos-2 cells, cultures derived from adipose tissue displayed a level of staining more similar to adipose derived cultures that received vehicle alone than differentiated osteoblasts. In order to compare the level of osteogenic differentiation between groups, the amount of alizarin red staining was assessed by eluting the dye retained by each sample. The absorbance of the dye elutant was subsequently measured at 550 nm (Figure 2.3 a, b). The dye elution data were analysed using the unpaired Student's T-Test with a one-tailed distribution. A large significant increase in the amount of alizarin red occurred between Saos-2 cultures that received treatment and those that did not (p-value > 0.0001). No difference was observed between adipose derived SVF cultures that received differentiation medium and those that received vehicle (p-value 0.4019). Considering the above, it is likely that the adipose derived cultures obtained using collagenase digestion did not undergo osteogenic differentiation.



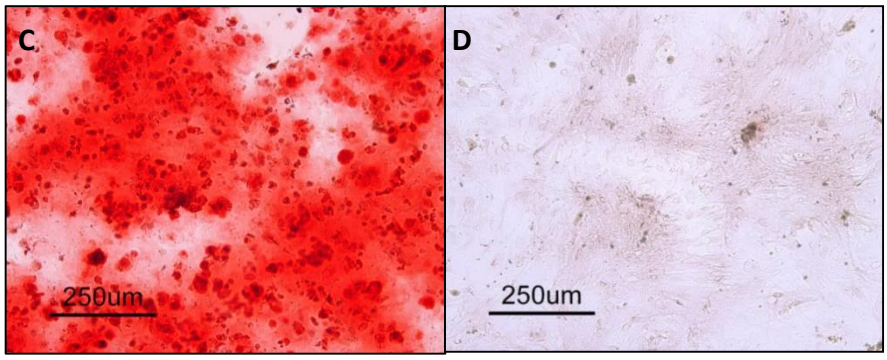


Figure 2.2. Alizarin red staining of adipose SVF cells and Saos-2 cells. Adipose SVF cells (b, d) and Saos-2 cells (a, c) received either osteogenic medium for 21 days (c, d) or growth medium supplemented with vehicle control for 5 days (a, b). Following culture, cells were fixed and stained with alizarin red. Images taken at 10x.

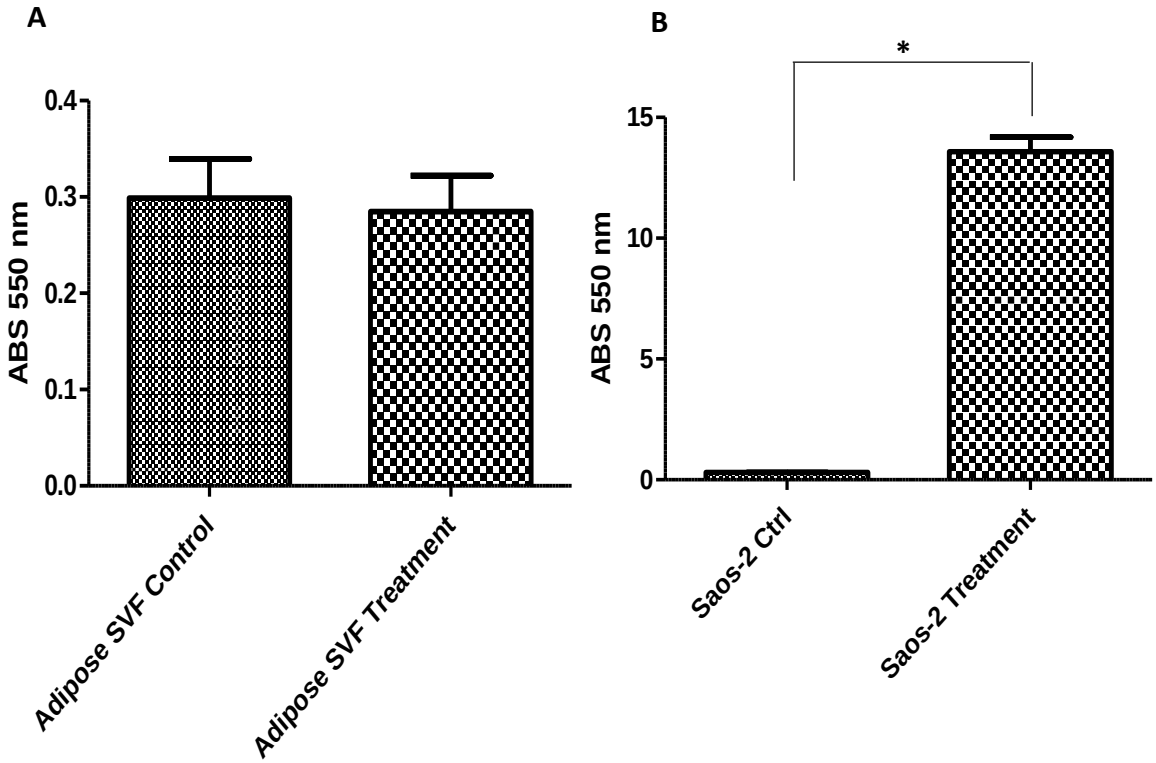


Figure 2.3. Alizarin red stain elution of adipose SVF cells following magnetic isolation (A) and Saos-2 cells (B). Absorbance read at 550 nm of alizarin red eluted from adipose SVF and Saos-2 cells cultured in osteogenic differentiation media (Treatment) or control media (Ctrl). Data points represent measurements made in triplicate, +/- standard error. Data points labelled treatment represent samples diluted in CPC at a ratio of 1:3, the absorbance values of these samples were then multiplied by a factor of 3. Saos-2 data point labelled treatment represents samples diluted in CPC at a ratio of 1:10, the absorbance values of these samples were then multiplied by a factor of 10. Individual cultures are used as replicates, where n=9 for adipose derived cultures and n=3 for Saos-2 cultures.* indicates a significant difference ($P < 0.05$).

2.3.3 Adipogenic differentiation of adipose derived SVF cells

Previous research has demonstrated that a combination of human insulin, biotin, IBMX pantothenate, dexamethasone, and rosiglitazone supplemented into growth medium is sufficient to differentiate MSCs into adipocytes. In order to determine if rat adipose-derived cells were capable of adipogenesis, cultures were treated with the above supplements for 12 days. Furthermore, the mouse pre-adipocyte cell line, 3T3-L1, is often used as a model of *in vitro* adipogenesis. As a positive control for adipogenic differentiation cultures of 3T3-L1 cells received identical treatment conditions as rat adipose derived cells. Following the culture period, cells were fixed and stained with oil red o to detect the production of lipid droplets within cells. 3T3-L1 cells (n=3) treated with adipogenic medium displayed a high level of staining, indicating a robust differentiation response (Figure 2.4 c). In comparison, adipose derived cells (n=8) displayed a low level of staining, with only a small number of positively stained cells observed (Figure 2.4 d). In order to further compare the level of adipogenic differentiation between groups, the amount of oil red o staining was assessed by eluting the dye retained by each sample. The absorbance of the dye elutant was subsequently measured at 540 nm (Figure 2.5). The dye elution data were analysed using the unpaired Student's T-Test with a one-tailed distribution. A significant difference in the level of staining between control and treatment cultures of 3T3-L1 cells was observed (p-value > 0.0001). However no statistical difference between adipose-derived treatment and control cultures was observed (p-value 0.1774).

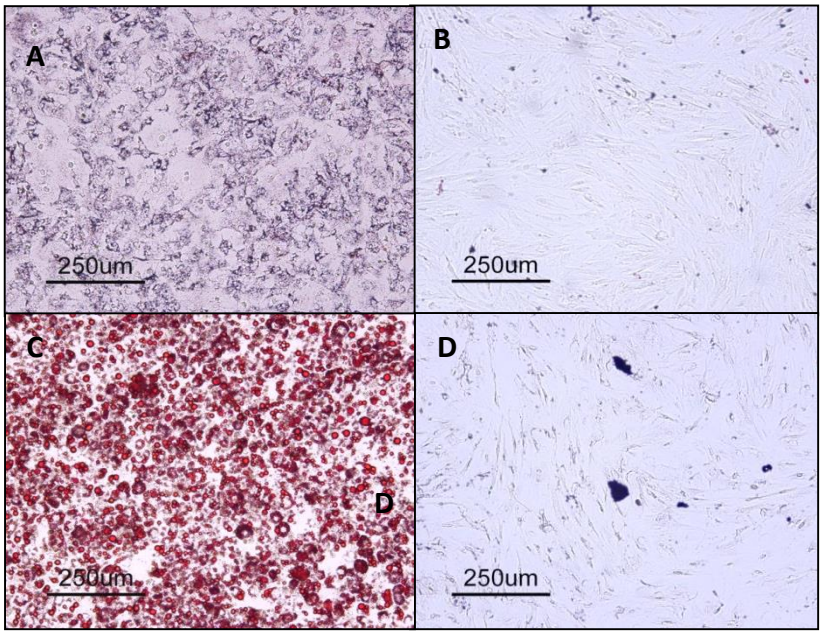


Figure 2.4. Oil red o staining of adipose SVF cells and 3T3-L1 cells. 3T3-L1 cells (a, c) shown as positive control. Cells received either adipogenic medium for 12 days (c, d) or growth medium supplemented with vehicle for 6 days (a, b). Following treatment period cells were fixed and stained with oil red o. Images taken at 10x magnification.

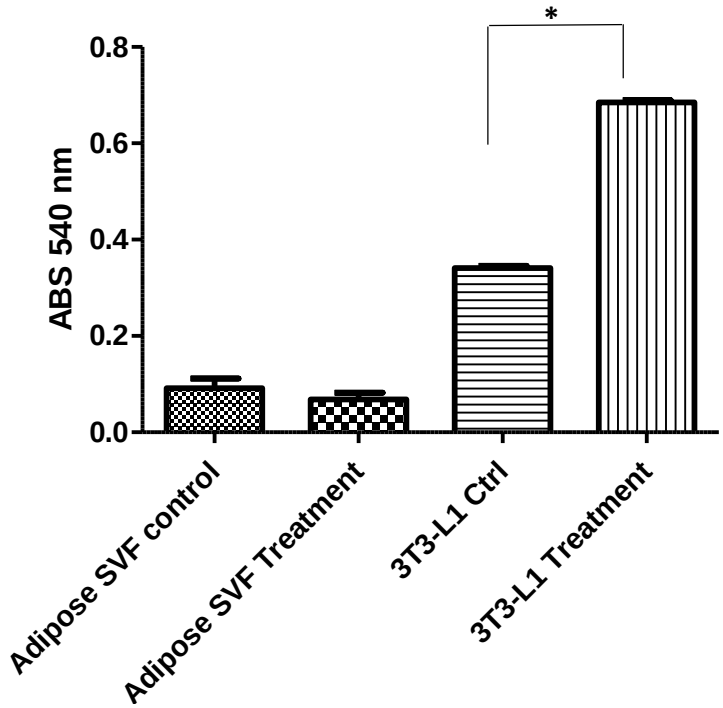


Figure 2.5. Oil red o stain elution of adipose SVF cells and 3T3-L1 cells. Absorbance read at 540 nm of oil red o eluted from adipose SVF and 3T3-L1 cells cultured in adipogenic differentiation media (Diff) or control media (Ctrl). Data points represent measurements made in triplicate, +/- standard error. Individual cultures are used as replicates, where n=8 for adipose derived cultures and n=3 for 3T3-L1 cultures * indicates a significant difference ($P < 0.05$).

2.3.4 Effect of removal of non-MSC adipose SVF cells from cell population on osteogenic differentiation

In order to improve differentiation of adipose derived cells, magnetic based immunological cell sorting was conducted to increase the proportion of MSCs *in vitro* by removing of CD31 positive endothelial cells, CD54 positive fibroblasts and CD45 positive hematopoietic cells. During the separation procedure, the total number of cells was counted following the removal cells expressing each marker (Figure 2.6). Furthermore, cells were also isolated from bone marrow, in order to establish cultures with known differentiation capacities. For both adipose (Figure 2.6 a) and bone marrow (Figure 2.6 b) the total number of cells decreased after each incubation event. This suggests that the cell cultures subjected to magnetic sorting possessed fewer non-MSC SVF cells at the end of the procedure.

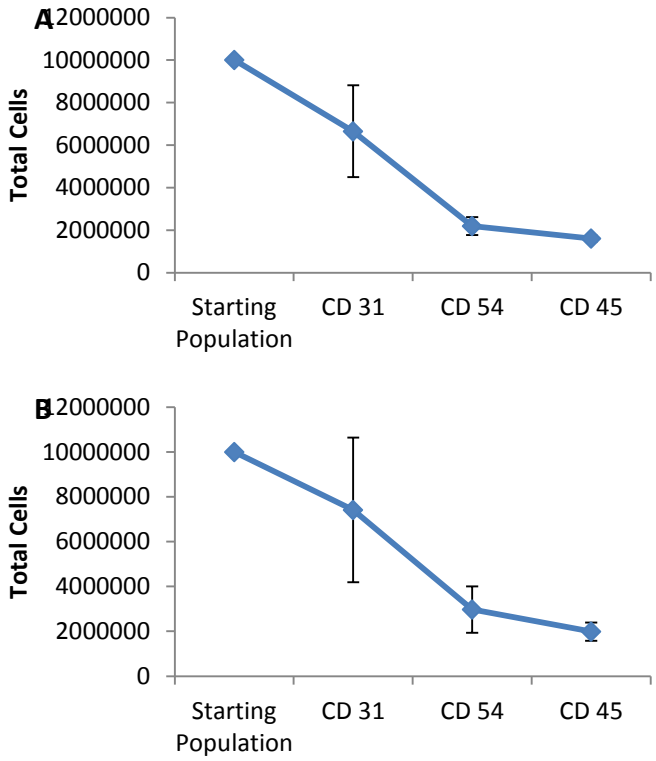


Figure 2.6. Cell yields following serial magnetic separation**.** Adipose SVF cells (a) and bone marrow SVF cells (b) were counted following the serial removal of CD 31 positive cells, CD 54 positive cells, and CD 45 cells. Data points acquired from 3 biological replicates. The starting population was diluted to $1x10^6$ cells for each trial.

In order to determine if magnetic sorting affected the osteogenic capacity of adipose derived cultures, sorted (n=6) and non-sorted cells (n=9) were cultured in the presence of osteogenic media for 21 days (Figure 2.7 e, f). Sorted and non-sorted cultures exposed to osteogenic media (figure 2.7 e, f) showed a similar level of alizarin red staining to vehicle controls (Figure 2.7 b, c). Additionally, adipose derived cells that received osteogenic media displayed much less staining than differentiated Saos-2 cells (n=3). In order to compare the level of osteogenic differentiation between groups, the amount of alizarin red staining was assessed by eluting the dye retained by each sample. The absorbance of the dye elutant was subsequently measured at 550 nm (Figure 2.8 a, b). The dye elution data were analysed using the unpaired Student's T-Test with a one-

tailed distribution. No difference was observed between non-sorted cultures that received differentiation medium and those that received vehicle (p-value 0.4019). Similarly, no differences were observed between sorted cultures and corresponding vehicle controls (p-value 0.0628). A large significant increase in the amount of alizarin red occurred between Saos-2 cultures that received treatment and those that did not (p-value > 0.0001). Furthermore, no difference was observed between sorted and non-sorted treatment groups (p-value 0.2812).

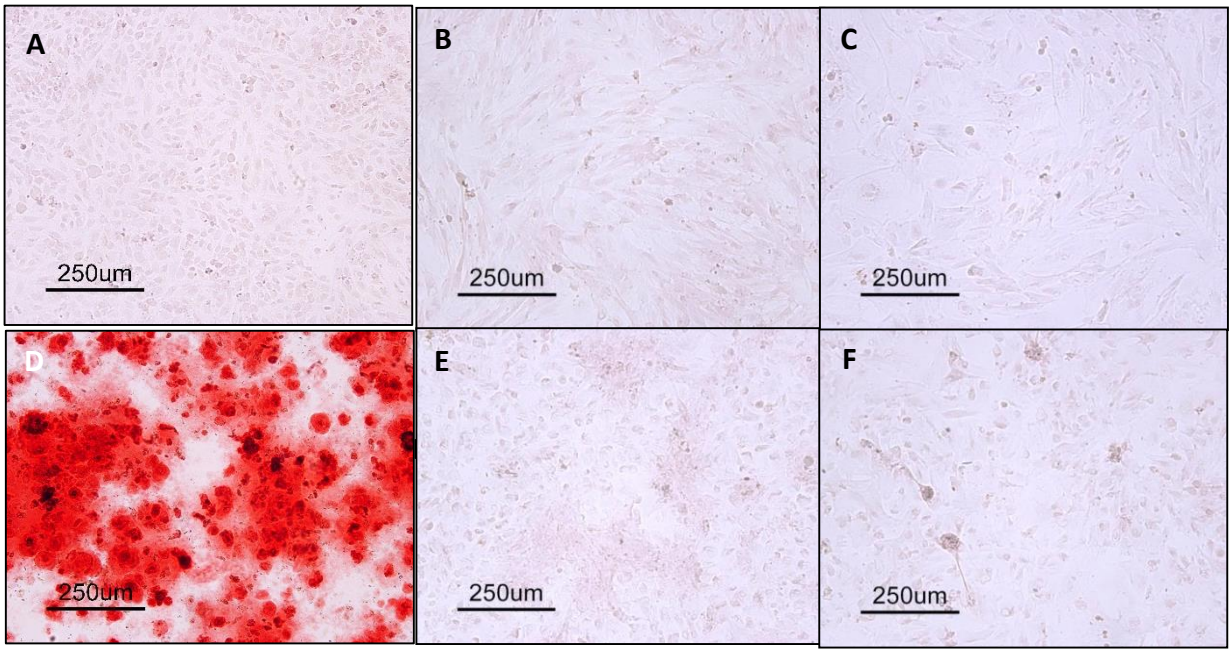


Figure 2.7. Alizarin red staining of adipose SVF cells following magnetic isolation. Adipose SVF cells were subjected to immunological separation (c, f) or excluded from the separation procedure (b, e). Saos-2 cells (a, d) shown as positive control. Cells received either osteogenic medium for 21 days (d, e, f) or growth medium supplemented with vehicle for 5 days (a, b, c). Following treatment period cells were fixed and stained with alizarin red. Images taken at 10x magnification.

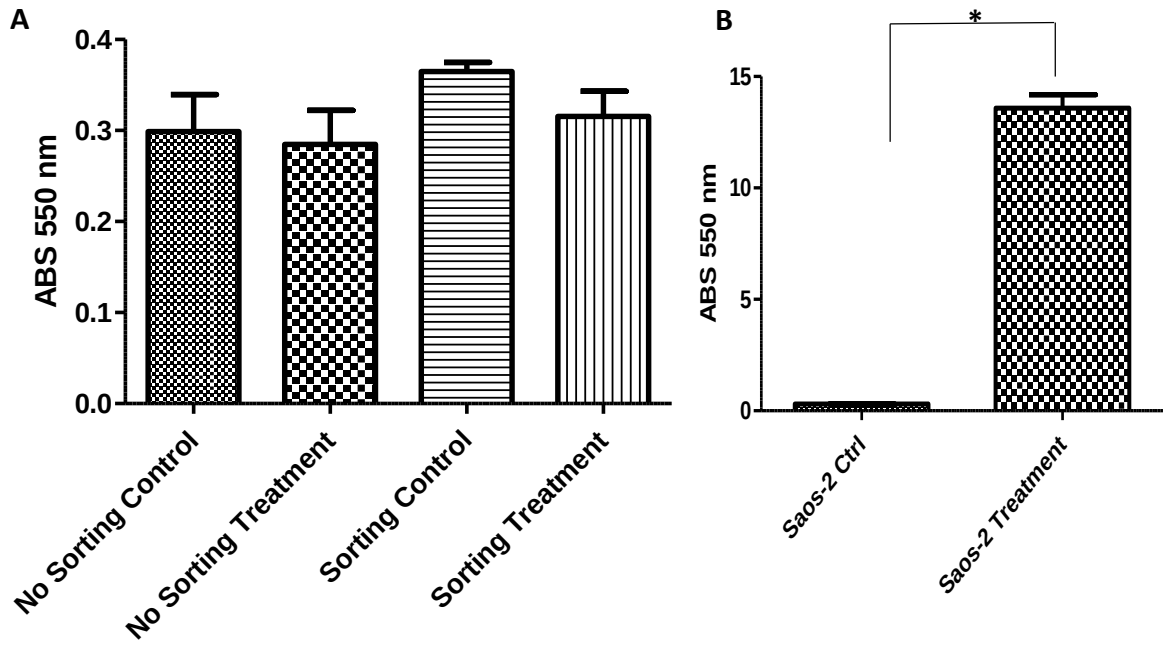


Figure 2.8. Alizarin red stain elution of adipose SVF cells following magnetic isolation (A) and Saos-2 cells (B). Absorbance read at 550 nm of alizarin red eluted from adipose SVF and Saos-2 cells cultured in osteogenic differentiation media (Diff) or control media (Ctrl). Adipose SVF cells were subjected to magnetic separation (no sorting) or excluded from the separation procedure (sorting control). Data points represent measurements made in triplicate, +/- standard error. Data points labelled treatment represent samples diluted in CPC at a ratio of 1:3, the absorbance values of these samples were then multiplied by a factor of 3. Saos-2 data point labelled treatment represents samples diluted in CPC at a ratio of 1:10, the absorbance values of these samples were then multiplied by a factor of 10. Individual cultures are used as replicates, where n=6 for sorted adipose, n=9 for non-sorted, and n=3 for Saos-2 cultures * indicates a significant difference ($P < 0.05$).

In order to determine if magnetic sorting affected the adipogenic capacity of adipose derived cultures, sorted (n=6) and non-sorted cells (n=9) were cultured in the presence of adipogenic media for 12 days (Figure 2.9). Sorted and non-sorted cultures exposed to adipogenic media (Figure 2.9 e, f) displayed small number of positively stained adipocytes when compared to vehicle controls (Figure 2.9 b, c). As a positive control for adipogenesis, 3T3-L1 pre-adipocytes (n=3) were subjected the same treatment and staining procedure as adipose derived cells. Pre-adipocytes that received treatment (Figure 2.9 a) showed a robust differentiation response when compared to 3T3-L1 cultures that received vehicle (Figure 2.9 b). Similar observations were made using phase

contrast microscopy (appendix Figure 1) In order to compare the level of adipogenic differentiation between groups, the amount of oil red o staining was assessed by eluting the dye retained by each sample. The absorbance of the dye elutant was subsequently measured at 540 nm (Figure 2.10). The dye elution data were analysed using the unpaired Student's T-Test with a one-tailed distribution. No difference was observed between non-sorted cultures that received differentiation medium and those that received vehicle (p-value 0.1774). Similarly, no differences were observed between sorted cultures and corresponding vehicle controls (p-value 0.2267). A large significant increase in the amount of oil red o occurred between 3T3-L1 cultures that received treatment and those that did not (p-value > 0.0001). Furthermore a small but significant increase was observed between sorted and non-sorted treatment groups (p-value 0.032).

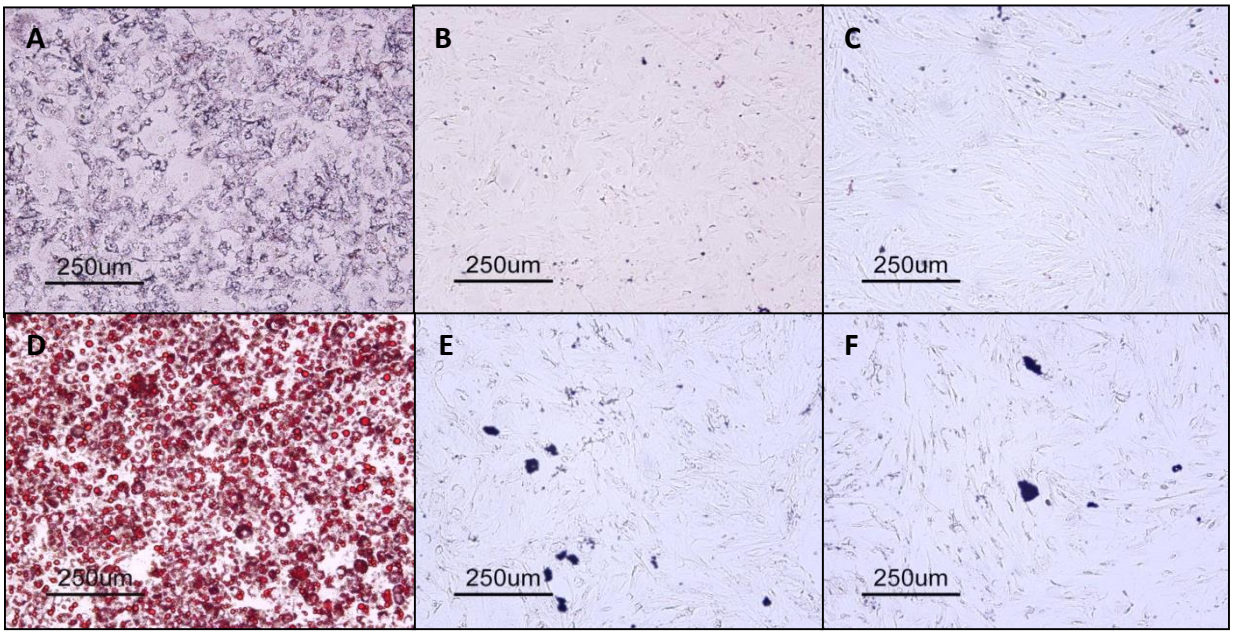


Figure 2.9. Oil red o staining of adipose SVF cells following magnetic isolation. Adipose SVF cells were subjected to magnetic separation (c, f) or excluded from the separation procedure (b, e). 3T3-L1 cells (a, d) shown as positive control. Cells received either adipogenic medium for 12 days (d, e, f) or growth medium supplemented with vehicle for 6 days (a, b, c). Following treatment period cells were fixed and stained with oil red o. Images taken at 10x magnification.

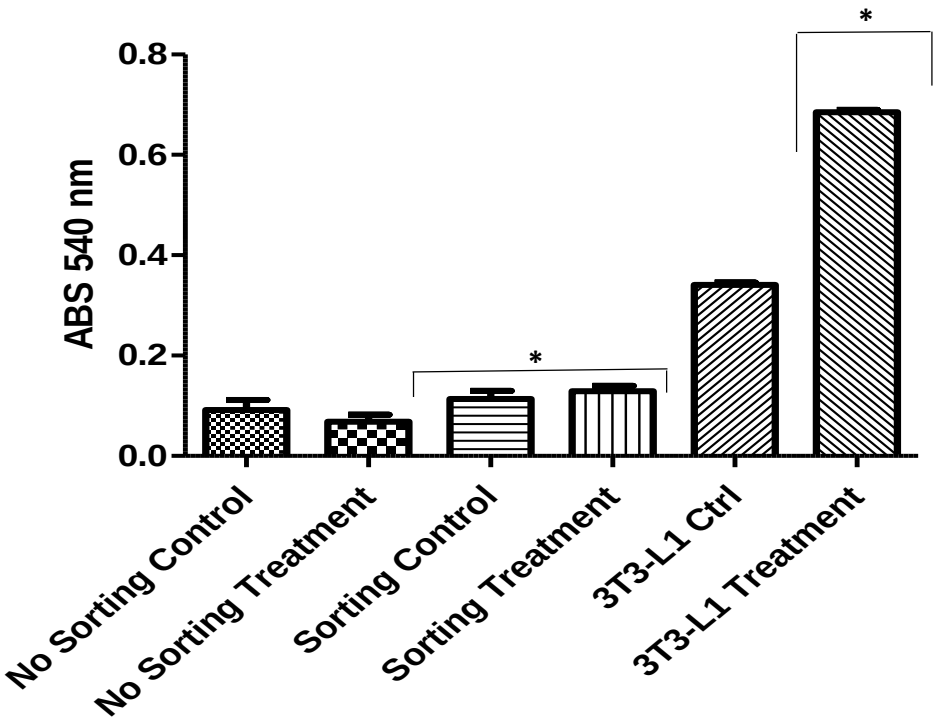


Figure 2.10. Oil red o stain elution of adipose SVF cells following magnetic isolation. Absorbance read at 540 nm of oil red o eluted from adipose SVF and 3T3-L1 cells cultured in adipogenic differentiation media (Diff) or control media (Ctrl). Adipose SVF cells were subjected to magnetic separation (no sorting) or excluded from the separation procedure (sorting control). Data points represent measurements made in triplicate, +/- standard error. Individual cultures are used as replicates, where n=6 for adipose sorted, n= 8 for adipose non-sorted, and n=3 for 3T3-L1 cultures * indicates a significant difference ($P < 0.05$).

2.3.5 Effect of removal of non-MSC bone marrow SVF cells from cell population on osteogenic differentiation

Compared to adipose, bone marrow is a better defined source of mesenchymal stem cells. In order to determine if the osteogenic and adipogenic treatments used on sorted and non-sorted adipose derived cells could induce a robust differentiation response, sorted and non-sorted bone marrow derived cells were subjected to treatments conditions identical treatments conditions as adipose derived cultures.

Sorted (n=9) and non-sorted (n=9) bone marrow cultures exposed to osteogenic media (Figure 2.11 e, f) showed a similar level of alizarin red staining to vehicle controls (Figure 2.11 b, c). Additionally, bone marrow derived cells that received osteogenic

media displayed much less staining than differentiated Saos-2 cells. In order to compare the level of osteogenic differentiation between groups, the amount of alizarin red staining was assessed by eluting the dye retained by each sample (Figure 2.12). Moreover, a small but significant difference in the amount of alizarin red eluted was observed between non-sorted cultures that received differentiation medium and those that received vehicle (p-value 0.0009). Similarly, a small difference was observed between sorted cultures and corresponding vehicle controls (p-value 0.0253). Furthermore no difference was observed between sorted and non-sorted treatment groups (p-value 0.1654).

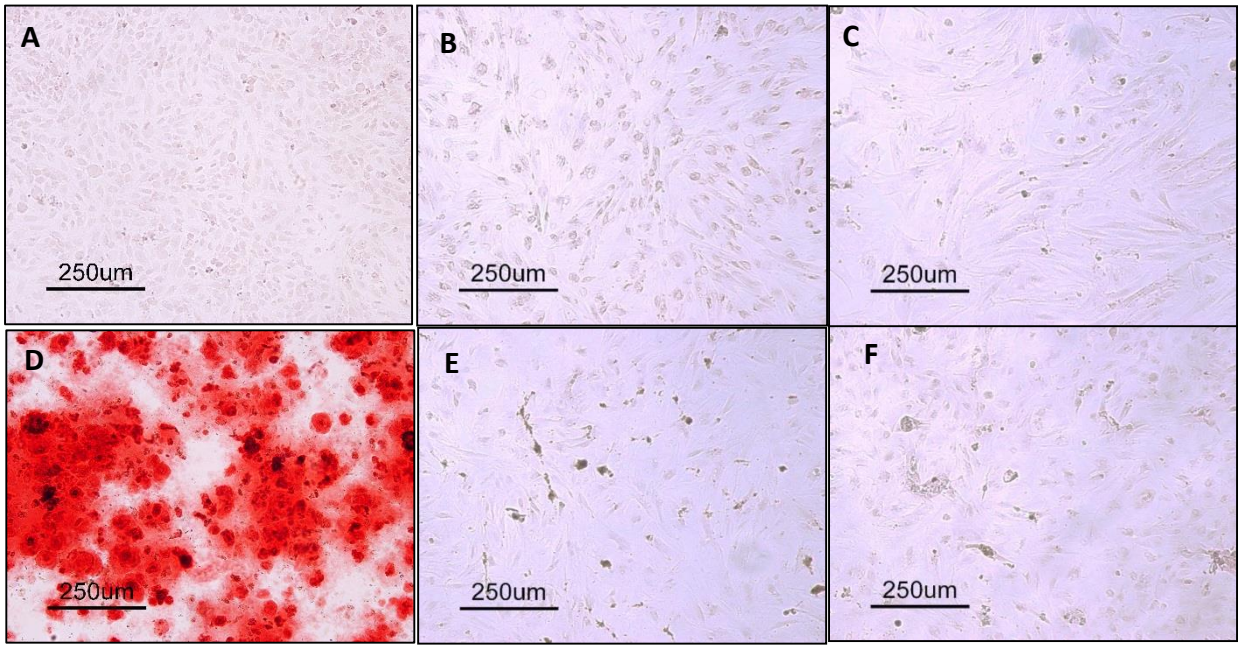


Figure 2.11 Alizarin red staining of bone marrow SVF cells following magnetic isolation. Bone marrow SVF cells were subjected to immunological separation (c, f) or excluded from the separation procedure (b, e). Saos-2 cells (a, d) shown as positive control. Cells received either osteogenic medium for 21 days (d, e, f) or growth medium supplemented with vehicle for 5 days (a, b, c). Following treatment period cells were fixed and stained with alizarin red. Images taken at 10x magnification.

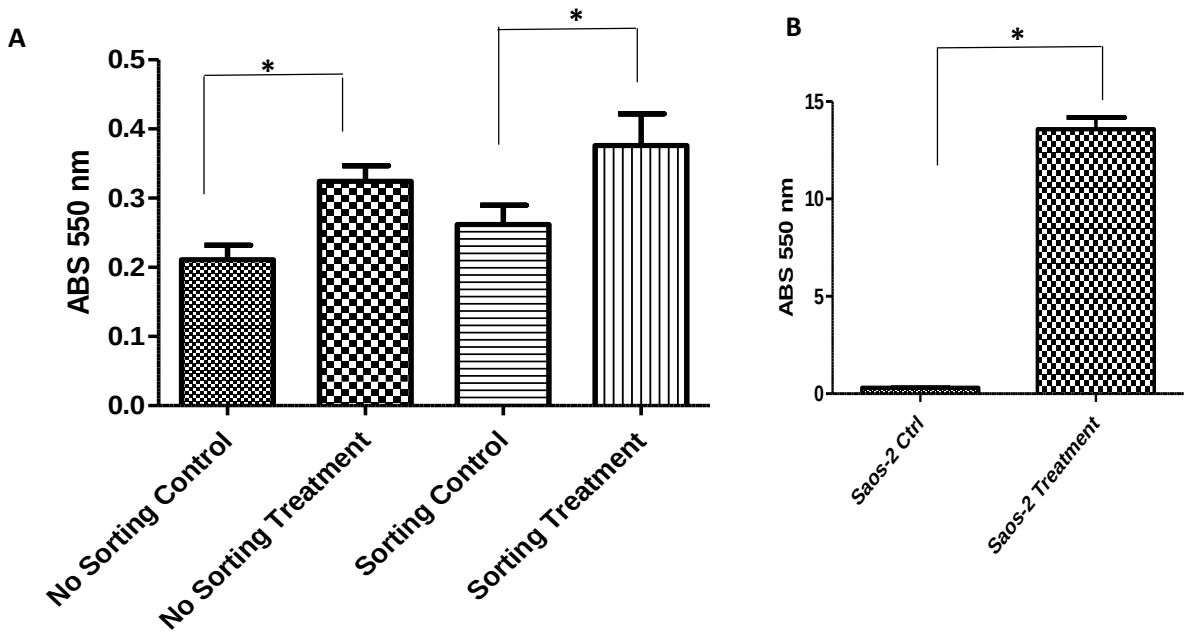


Figure 2.12. Alizarin red stain elution of bone marrow SVF cells following magnetic isolation (A) and Saos-2 cells (B). Absorbance read at 550 nm of alizarin red eluted from marrow SVF and Saos-2 cells cultured in osteogenic differentiation media (Diff) or control media (Ctrl). Adipose SVF cells were subjected to magnetic separation (no sorting) or excluded from the separation procedure (sorting control). Data points represent measurements made in triplicate, +/- standard error. Data points labelled treatment represent samples diluted in CPC at a ratio of 1:3, the absorbance values of these samples were then multiplied by a factor of 3. Saos-2 data point labelled treatment represents samples diluted in CPC at a ratio of 1:10, the absorbance values of these samples were then multiplied by a factor of 10. Individual cultures are used as replicates, where n=9 for marrow sorted, n= 9 for marrow non-sorted, and n=3 for Saos=2 cultures * indicates a significant difference ($P < 0.05$).

Sorted (n=9) and non-sorted (n=9) bone marrow derived cells were cultured in the presence of adipogenic media for 12 days (Figure 2.13). As assessed by phase contrast microscopy, sorted and non-sorted cultures exposed to adipogenic media (Figure 2.13 e, f) displayed no adipocytes as did vehicle controls (Figure 2.13 b, c).

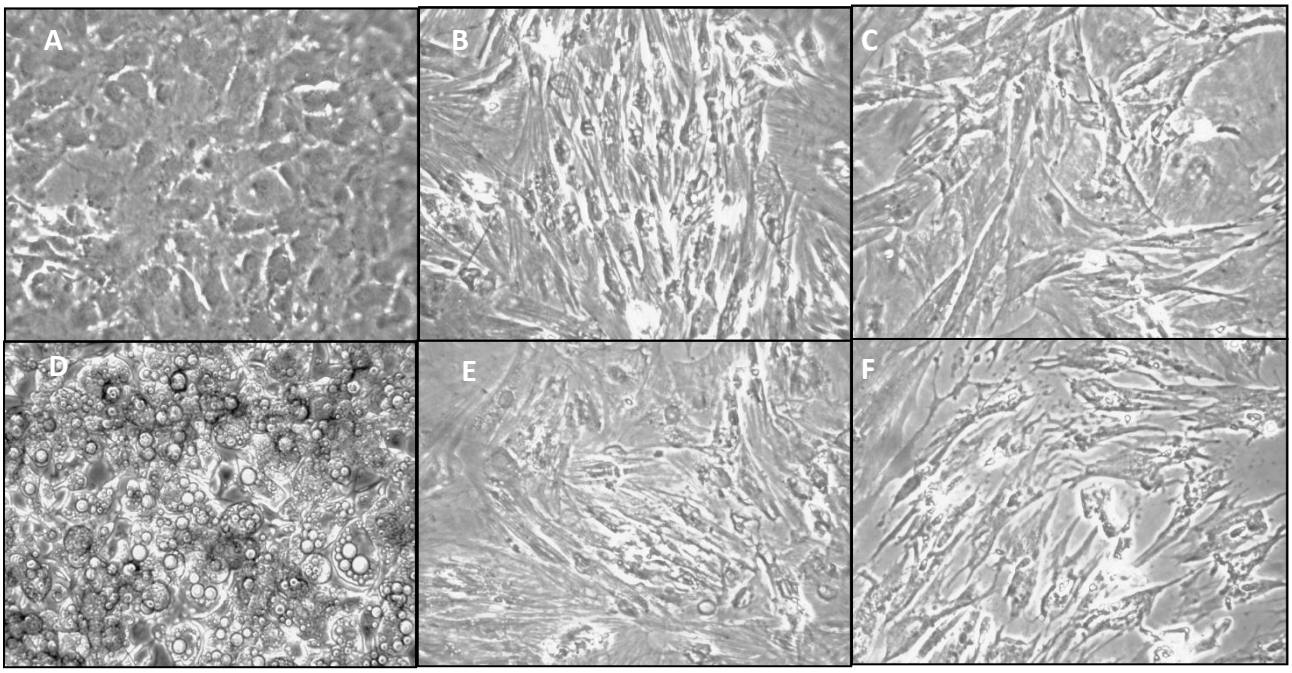


Figure 2.13. Phase contrast images of bone marrow SVF cells following magnetic isolation and adipogenic treatment. Adipose SVF cells were subjected to magnetic separation (c, f) or excluded from the separation procedure (b, e). 3T3-L1 cells (a, d) shown as positive control. Cells received either adipogenic medium for 12 days (d, e, f) or growth medium supplemented with vehicle for 6 days (a, b, c). Images taken at 20x magnification.

2.4 Discussion:

It was anticipated that access to human adipose tissue would be limited. Therefore, it was necessary to develop the methodology for isolating and differentiating stromal vascular derived MSCs using adipose obtained from rat. MSCs isolated from tissue possess the ability to proliferate *in vitro* (33). A 0.1% collagenase solution was

used to digest adipose and the cells obtained were cultured in DMEM/F-12 medium supplemented with 10% FBS. 2-4 days following seeding, it was observed that culture plates seeded with adipose SVF cells were ~ 80-90% confluent. The observed growth time is similar to the population doubling time of adipose derived SVF cells calculated by Tawonsawatruk et al. 2012. The authors isolated rat adipose SVF cells using collagenase digestion and calculated that the population doubled within 4.6 days (149). The above results demonstrate that the enzymatic digestion procedure used on rat adipose successfully isolated proliferative stromal vascular cells.

Osteogenic differentiation of MSCs *in vitro* can be achieved by exposure to dexamethasone, ascorbic acid, and β-glycerophosphate. When provided together, these compounds activate the expression of genes which induce MSCs to produce a calcified extracellular matrix and differentiate into osteoblasts (148). In order to determine if adipose derived SVF cultures contained cells with osteogenic capacity, these cells were incubated in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate for 21 days. Comparison of treated cells with differentiated osteosarcoma cells using alizarin red staining showed that the isolated rat SVF cells did not produce a calcified matrix, suggesting these cells did not undergo osteogenic differentiation. Additionally, separate cultures of cells were treated with adipogenic medium. First, SVF cells were cultured in the presence of insulin, biotin, IBMX, pantothenate, dexamethasone, and rosiglitazone for 3 days in order to induce MSCs to commit to the adipocyte lineage and develop into pre-adipocytes. Subsequently, the cells were exposed to the same combination of compounds except for rosiglitazone and IBMX for 9 days in order to induce the production of lipid droplets within cells and differentiation into multi-ocular adipocytes

(150). Following the treatment period, a minute adipogenic response was observed such that only a few adipocytes were produced. The osteogenic and adipogenic treatments used have been previously demonstrated to induce differentiation of adipose derived MSCs (151, 152). Therefore, it is unlikely that a large population of naïve MSCs was present in the isolated SVF cell cultures obtained from rat adipose.

Considering that MSCs represent a small population of total SVF cells it was hypothesized that removal of non-MSC cells would increase the proportion of cells capable of differentiation. In order to enrich cultures, SVF cells were incubated with antibody-bound magnetic beads in order to remove CD 54 positive fibroblasts, CD 31 positive endothelial cells, and CD 45 positive cells of hematopoietic origin. These sorted cultures were subsequently exposed to osteogenic or adipogenic differentiation medium. Alizarin red staining demonstrated osteogenic differentiation did not occur in sorted cultures. Similarly, oil red o staining demonstrated that only minimal adipogenesis occurred in enriched cultures, suggesting that removal of non-MSC cell types did not improve the ability of adipose derived SVF cells to differentiate. A small but significant increase in the amount of oil red o retained by sorted cultures treated with adipogenic medium compared to non-sorted cultures was observed. However, neither treatment group was significantly different from corresponding negative controls, suggesting that sorting did not increase the ability of SVF cells to undergo adipogenesis. In order to obtain a sufficient number of cells for the magnetic-sorting method, cells were subcultured for 10 passages. Investigations into the effect of long-term culture on MSCs suggest that differentiation capacity negatively correlates with time in culture. For example, Noer et al. 2007 compared the osteogenic capacity of freshly isolated human

adipose derived SVF cells to cells of the same origin following 44-48 population doublings (153). Using alizarin red staining, the authors observed that long-term cultures possessed reduced osteogenic ability compared to fresh cultures. Similarly, Kretlow et al. 2008 compared adipogenic ability of freshly isolated murine bone marrow SVF cells to cells of the same origin following 6 passages. Using oil red o staining Kretlow et al. observed that adipogenic treatments were less effective at inducing differentiation if cells were cultured for a greater length of time (154). With this in mind, it is plausible that SVF cultures did not experience differentiation do to prolonged time in cultures. Furthermore, it was observed that the sorting procedure reduced the number cells following sequential incubations with antibody-bound beads. This suggests that non-MSC cells were removed. It is also plausible that cell death occurred during this procedure and therefore enrichment never occurred or was limited. In order to elucidate this possibly fluorescent activated cell sorting would be required. However, the arrival of human tissue was not under the control of myself. Considering this, the implementation of these procedures was anticipated to increase the length of this preliminary study and therefore causing a possible conflict with the ability to begin experimentation on human samples.

Bone marrow stromal cell differentiation has been thoroughly studied since the 1970's (155). Compared to adipose, which was discovered to possess MSCs by Zuk et al. in 2001(28), bone marrow is a better characterized source of stem cells. Considering that adipose derived MSCs did not display a robust differentiation response using standard differentiation procedures, it was desirable to test these same methods on bone marrow derived SVF cells. Moreover, similar to adipose, bone marrow contains

fibroblasts, endothelial cells and cells hematopoietic origin (156). Therefore, in order to determine whether magnetic could increase the proportion of isolated MSCs *in vitro*, sorting was also performed on marrow SVF cells. Following osteogenic treatment, bone marrow SVF cells were stained with alizarin red. Spectrophotometric analysis of eluted dye revealed a small significant increase in the amount of dye retained by sorted and non-sorted cultures compared to corresponding controls. Treatment cultures not subjected to sorting increased by a magnitude of 0.1136 while sorted treatment cultures increased by 0.1141. Compared to differentiated osteosarcoma cells which experienced an increase in dye retention by 13.28, the increased staining of bone marrow cells is minute and not likely indicative of differentiation. Additionally, phase contrast microscopy of sorted and non-sorted bone marrow SVF cells treated with adipogenic media revealed that the minimal adipogenic response occurred, similar to that of adipose derived cells. Like adipose derived SVF cells, marrow cells were cultured for 10 passages. Thus, the most plausible reason that these cells did not experience a robust differentiation response is prolonged time in culture.

In addition to osteogenic and adipogenic differentiation, MSCs are characterized by the ability to differentiate along the chondrogenic lineage into cartilage forming chondroblasts. Chondrogenic differentiation of cultured stem cells is typically achieved by centrifugation of cells into a pellet and subsequent treatment with insulin, transferrin, linoleic acid, dexamethasone, and transforming growth factor β. Differentiated chondroblasts produce sulfated proteoglycans in the extracellular matrix, which can be detected with alcian blue staining (157, 158). It was desirable to develop methods of

inducing chondrogenesis using rat derived cells. However, due to the arrival of human samples further work on rat samples could not be performed.

2.5 Conclusions

This study demonstrated that the collagenase digestion method used successfully isolated proliferative cells from adipose tissue. When treated with differentiation medium these did not display robust adipogenic or osteogenic responses. In order to increase the number of MSCs *in vitro,* magnetic sorting was performed to remove non-MSC SVF cells. It was observed that sorting had no effect on differentiation. Furthermore, rat bone marrow SVF cells were subjected to sorting and subsequent incubation in differentiation mediums. Neither sorted nor non-sorted bone marrow cells experienced a strong differentiation response. It is plausible that sorting induced cell death cells during isolation. Therefore future work should assess this possibility using fluorescent activated cell sorting. Furthermore, MSCs *in vitro* lose differentiation potency with prolonged time *in vitro*. It is likely that this phenomenon effected SVF cells obtained from adipose and marrow. Therefore, future work on human adipose will implement an experimental design that minimizes the time cells spend *in vitro* prior to differentiation.

Chapter 3: Human Adipose Derived Mesenchymal Stem Cells:

Collagenase versus Sonication based Isolation

Objectives: Determine if the sonication based method is capable of isolating mesenchymal stem cells from human adipose tissue with an efficiency comparable to the collagenase based method.

Hypothesis: The sonication based will allow the isolation of cells capable of *in vitro* differentiation into osteoblasts, adipocytes, and chonrdroblasts.

Abstract

Adipose derived MSCs possess the ability to differentiate into connective tissues as well as the capacity to enhance cartilage and bone regeneration. Yet, for experimental and therapeutic purposes, MSCs have been traditionally isolated from adipose using the digestive enzyme, collagenase, which cannot enter a patient. With this issue in mind non-enzymatic sonication based method of MSCs isolation was compared to the collagenase method. Excised human adipose from three patients was processed using either collagenase or sonication methods. Adherent, proliferative stromal cultures were obtained from all individuals using collagenase and cultures from 2 patients were obtained using sonication. Compared to the collagenase method, sonication yielded fewer total cells. However, when provided osteogenic media, alizarin red staining for osteoid revealed that sonication derived cultures possessed a higher capacity for bone differentiation than those obtained using collagenase. Following adipogenic treatment, oil red o staining for lipid accumulation was similar in cultures obtained using either isolation method. Moreover, following incubation in the presence of chondrogenic medium, ImageJ analysis of alcian blue staining showed that collagenase derived stromal cells underwent a small level of chondrogenisis, while cells obtained from sonication did not show a greater level of staining compared to negative controls. These results suggest that it is unlikely that sonication yielded multi-potent MSCs from adipose tissue. Importantly, sonication yielded cells with high osteogenic capacity and therefore has potential to be incorporated in therapies for bone repair.

3.1 Introduction:

Aging in humans is characterized by a functional decline due to histological and biochemical changes in tissue and organ systems. These functional declines often manifest as pathological conditions associated with the elderly, such as heart failure, osteoporosis, and arthritis. Furthermore, almost all tissues show an age-related decline in cellular turnover and regeneration in response to injury or stress (159, 160). Stem cells are undifferentiated cells which divide into one cell that retains its undifferentiated character, and a second cell that can undergo one or more paths of differentiation. Adult stem cells are found in the tissues of organs after the organ has matured. Such cells are rare and function to replace dead or dysfunctional cells and repair damaged tissues (5). Considering the plethora of pathologies that are associated with aging, stem cell biology has been intensely researched with the aim of developing therapies capable of reversing degenerative conditions.

Mesenchymal stem cells (MSCs) are a population of adult stems cells found in bone marrow, adipose, and other connective tissues which contribute to tissue regeneration. MSCs isolated from tissues are isolated as part of a stromal vascular fraction (SVF) which consists of other stromal cells such as hematopoietic cells, and connective tissue cells such as fibroblasts and endothelial cells (33). Stem cell therapy utilizing isolated MSCs derived from aspirated bone marrow has been demonstrated to enhance the repair of damaged tissue such as bone, cartilage, and cardiac tissue (9-11)

In vitro, MSCs are characterized as tissue culture plastic adherent cells able to differentiate into adipocytes, osteoblasts, and chondrocytes (33). Each of these differentiation lineages can be achieved by cell culture mediums containing chemical cocktails. Osteogenic differentiation of MSCs requires incubation with ascorbic acid, glycerophosphate, and dexamethasone resulting in an increase in calcium deposition in the extracellular matrix. Chondrogenic differentiation is performed by centrifuging cells to form a cell pellet and culturing the cell pellet with transforming growth factor beta, dexamethasone, ascorbic acid, and ITS+ (insulin, transferring, selenous acid and linolic acid,). The high pressure micro-environment of the pellet is believed to amplify differentiation. This results in the production of sulphated proteoglycans and type II collagen. Furthermore, adipogenic differentiation of MSCs is achieved by culturing cells with medium supplemented with dexamethasone, insulin, isobutyl methyl xanthine, and rosiglitazone (148, 161, 157).

The aspiration of bone marrow for regenerative therapy is a painful process. However, adipose deposits are ubiquitous and easily accessible in large quantities via liposuction aspiration. Moreover, adipose tissue is routinely extracted from adults undergoing liposuction surgery, and is subsequently discarded (14). Taken together, adipose tissue is an attractive source of stem cells for the use in regenerative medicine. The standard protocol for isolating MSCs uses bacterial derived collagenase to breakdown extracellular peptides in tissue (146). The method is not ideal for clinical applications because the sample will be contaminated with potentially toxic exogenous enzyme that cannot enter a patient. Recently, the company RegenaStem has patented a new method for isolating adipose derived stem cells. The method is based on mechanical

isolation of the cells using ultrasonic waves emitted from a standard tissue sonicator. Thus, a non-enzymatic method of isolating MSCs from adipose offers a potential therapeutic alternative to bone marrow based stem cell therapy.

3.2 Materials and Methods:

3.2.1 Reagents:

3-isobutyl-1-methylxanthine (IBMX), ascorbate-2-phosphate seqimagnesium salt hydrate, biotin, β-glycerophosphate disodium salt, bovine serum albumin (BSA) fraction V, cetylpyridium chloride, dexamethasone, d- pantothenate, dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), high glucose Dulbecco's modified eagles media (DMEM) powdered media, human recombinant insulin, paraformaldehyde, penicillin/ streptomycin, rosiglitazone, and 0.25% trypsin-EDTA were purchased from Sigma Aldrich.

Calcium chloride, glutamine, HEPES, methyl salicylate, paraplast X-TRA tissue embedding medium (paraffin), potassium chloride, potassium diphosphate, monobasic potassium phosphate, sodium bicarbonate, sodium chloride, sodium hydroxide and dibasic sodium phosphate were purchased from Fischer Scientific.

DMEM/ F-12 powdered media with L-glutamine, and Ham's F-12 powdered media with L-glutamine were purchased from Invitrogen. DPX mounting medium and formaldehyde were purchased from BDH chemicals. Collagenase type I was purchased from Worthington Biochemical. Hydrochloric acid, isopropanol, sodium hydroxide, and xylene were purchased from Caledon. Alizarin red, oil red O, and alcian blue 8GX were

purchased from Santa Cruz Biotechnology. ITS+ supplement was purchased from Cyagen Biosciences. TGFβ1 was purchased from Prospecbio.

3T3-L1 cell line was purchased from Eton Biosciences. Saos-2 cell line was a gift from the Dr. Ward lab (Brock University, St. Catharines, Ontario, Canada).

3.2.2 Sample Donors:

Excised adipose tissue from three female donors was obtained with approval of the Brock University research ethics board and informed consent of all patients. Patient information is summarized in Table 3.1.

Table 3.1: Patient Information

Donor	Gender	Age	BMI	Surgery
Patient 1	Female	36 yr	24.6	Abdominoplasty
Patient 2	Female	51 yr	25	Belt Lipectomy
Patient 3	Female	26 yr	-	Belt Lipectomy

3.2.3 Cell Isolation:

Adipose derived stromal vascular fraction (SVF) cells were isolated from excised human adipose tissue and subjected to either collagenase digestion or sonication. *Collagenase*: preformed as in methods 2.2.3. *Sonication*: adipose tissue sonication was performed using the UP400S (400 W, 24 kHz) sonicator and S14 sonotrode (Hielscher Ultrasonics) set to 0.4- 0.5 cycle and 50% amplitude. Tissue was placed on the ultrasonic probe of the device and exposed to ultrasonic waves for 30 second bursts. Samples were then centrifuged at 600 *x g* for 5 min. The supernatant containing the oil layer was

removed. Cells were re-suspended in growth media and centrifuged at 600 *x g* for 5 min. The cells were re-suspended in growth media and placed through a Sterileflip 100 μm filter (Milipore). The resultant cell suspension was placed in 175 cm^2 tissue culture treated flasks.

3.2.4 Cell Culture:

Adipose derived stromal vascular fraction cells: cells (passage 0) obtained using collagenase or sonication based methods were cultured in growth media at 37˚C, 5% CO_2 and 3% O_2 until cultures reached ~70-80% confluence (5-14 days). In order to avoid cell loss, media was changed 5-7 days after isolation. Following this period, media was changed every 2 days. Passage 0 cells were sub-cultured to passage 1 using 0.25% trypsin-EDTA solution and placed on 100 mm tissue culture treated plates (Sarstedt) at a density of 5000 cells/cm^2 and allowed to reach 70-80% confluence. Passage 2 cells were either stored in freezing media consisting of 10% DMEM/F-12, 10% DMSO, and 80% FBS and stored in liquid nitrogen or cultured at a density of 10 000 cells/cm^2 on 35 mm tissue culture treated plates (NEST). Passage 2 cells at 70-80% confluence were transferred to a 20% O_2 incubator and were treated with differentiation treatments or corresponding vehicles. Osteogenic and adipogenic treatments were performed as in methods 2.2.3. Osteosarcoma and preadipocytes were cultured as in methods 2.2.3.

3.2.5 Cell Counting:

Cell counts were performed using a haemocytometer. From passage 1 to passage 2 population doubling time was calculated using the formula: Doubling time = (T) (ln 2/ln (Nf/Ni), were T= elapsed time between passages, Nf = final cell number, Ni = initial cell number.

3.2.6 Staining:

Alizarin red and oil red o staining was performed as in methods 2.2.5. *Alcian Blue*: cell pellets were fixed overnight in 10% neutral buffered formalin. Pellets were dehydrated with a series of increasing ethanol for 30min each (30%, 50%, 70%, 80%, 95%) and placed in 100% ethanol twice for 45 min. Pellets were then cleared with methyl salicylate overnight. Pellets were then incubated at 80^{o}C in 50% methyl salicylate, 50% molten paraffin for 30min followed by two incubations in molten paraffin at 80^{o}C for 30 min. The cell pellets were then placed in embedding trays containing molten paraffin, allowed to cool and stored at 4^{o}C. Pellets were then cut into 10 micometer thick sections using an AO spencer 820 rotary microtome. Sections were then floated on warm water covering Starfrost saline coated slides (Sigma Aldrich). Following sectioning, water was removed from slides. Sections were deparafinized in xylene for 3 min and subsequently rehydrated in decreasing ethanol (100%, 95%, 70%, 50%) for 3min each. Slides were placed in distilled water for 3 min followed 3 min in 0.1M HCl. Slides were then incubated in 1% alcian Blue (dissolved in 0.1M HCl) for 10 min. Following staining, slides were briefly rinsed in 0.1M HCl and placed under running tap water for 10 min. Sections were then dehydrated with a series of increasing ethanol for 3 min each (50%, 70%, 95% 100%) and subsequently cleared in xylene for 3 min. Slides were then treated with DPX mounting media and cover-slipped (adapted from 158, 163).

3.2.7 Imaging:

Bright field images of cells in culture were captured using the Hund Wilovert S inverted microsocope from Fischer Scientific. Bright field images of stained cells and cell pellet sections were obtained using the Nikon eclipse 80i microscope.

3.2.8 Dye Elution:

Dye elution was performed as in methods 2.2.6.

3.2.9 ImageJ:

Images of stained sections were taken at 4x magnification using a Nikon eclipse 80i microscope under constant microscope settings. Images were then analysed using ImageJ densitometry and quantified as mean pixel density.

3.2.10 Statistics:

Dye elution and ImageJ data were analyzed using the Student's T-Test with a one tailed distribution. Doubling time data were analyzed using the Student's T-Test with a two tailed distribution. Data were analyzed on GraphPad Prisim version 5. $\alpha = 0.05$ was used to determined statistical significance. In each analysis, technical replicates (individual cultures) were treated as biological replicates in order to achieve statistical significance.

3.3 Results:

Collagenase and sonication based isolations were conducted on adipose tissue obtained from 3 patients. The cell suspension obtained from each isolation procedure was placed into one 175 cm^2 flask, resulting in a total of 6 flasks. In order to assess the ability of each method to isolate tissue culture plastic adherent cells, the number of adherent cells was counted once cultures reached ~70-80% confluence. The collagenase method yielded cells from each tissue sample. The sonication method yielded cells from adipose tissue obtained from patients 2 and 3, but not patient 1. Additionally, the collagenase method yielded a higher number of cells in a shorter period of time from each tissue

sample compared to the sonication method (Table 2, Figure 1). The above results demonstrate that the sonication based method is capable of obtaining adherent cells and suggest that compared to the collagenase based method, sonication is less effective at isolating adherent cells.

A characteristic of MSCs i*n vitro* is self-replication. Thus, it is desirable that the sonication based method is capable of isolating cells that replicate at a similar rate as those isolated through collagenase digestion. In order to characterize the growth of the SVF cells isolated using each method, the duration of time it takes a population of cells to double in number was compared between isolation groups (Figure 2). The mean doubling time for the collagenase group (n=6) was 107.6 +/- 12.6 hrs and 100.1 +/- 22.7 hrs for the sonication group (n=4). The doubling time data were analysed using an unpaired student's T-Test with a two-tailed distribution with $\alpha = 0.05$. No significant difference in doubling time was found between groups (p-value: 0.7633), suggesting that cells isolated by both methods have similar growth rates.

Table 3.2: Yield of adipose derived SVF cells after passage 0. Individual cultures are used as replicates, where n=6 for collagenase group and n= 4 for sonication group.

Patient	**Isolation Method**	**Culture Time**	**Cell Number**
1	Collagenase	10 days	1500000
1	Sonication	-	0
2	Collagenase	10 days	2360000
2	Sonication	14 days	1260000
3	Collagenase	5 days	4940000
3	Sonication	10 days	1100000

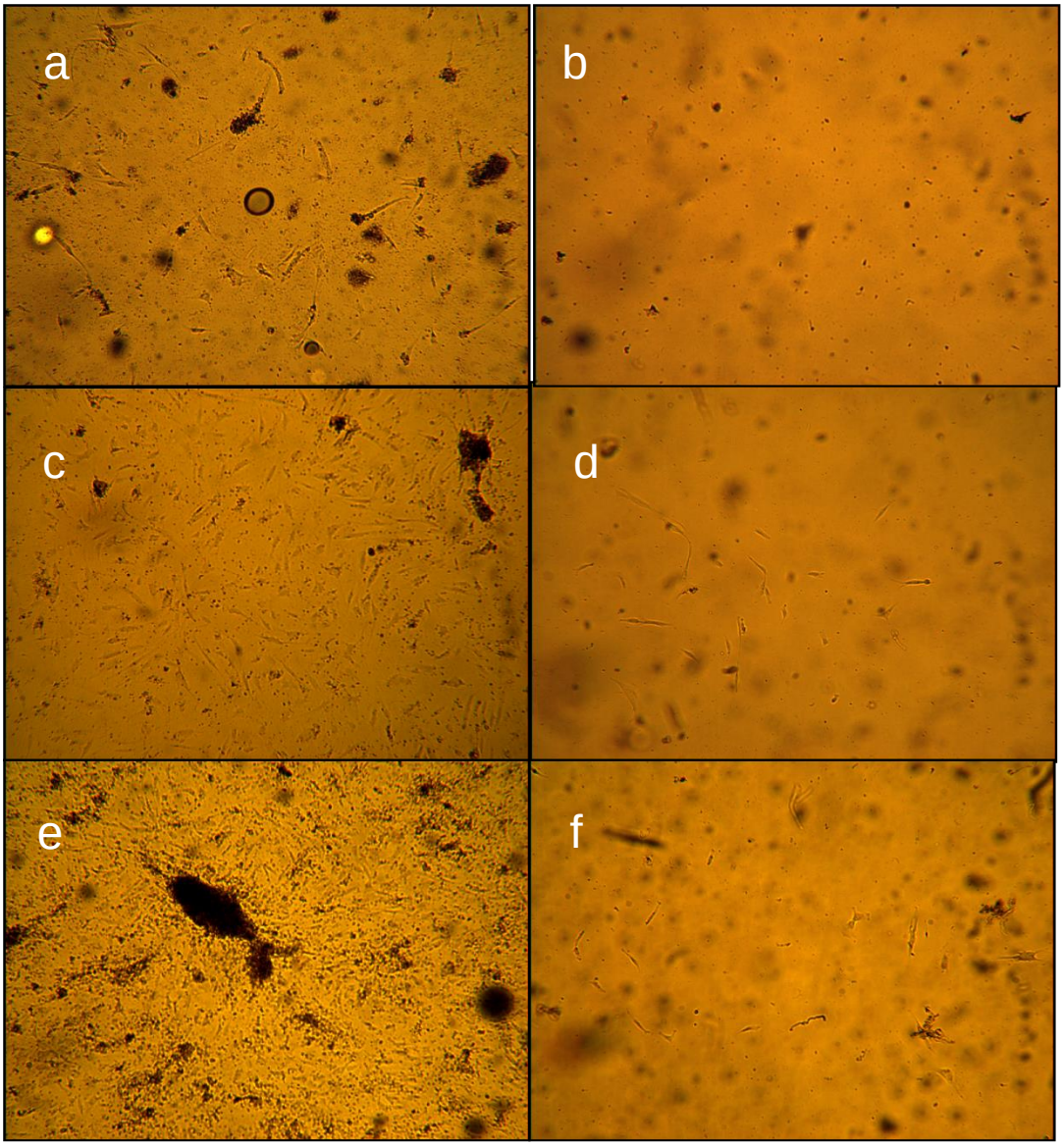


Figure 3.1. Adipose derived SVF cells after 5 days in culture at 4x magnification. Images obtained of cells isolated from patient 1, 2, and 3 using collagenase correspond to: a, c, and f, respectively. Images obtained of cells isolated from patient 1, 2, and 3 using sonication correspond to: b, d, and e, respectively.

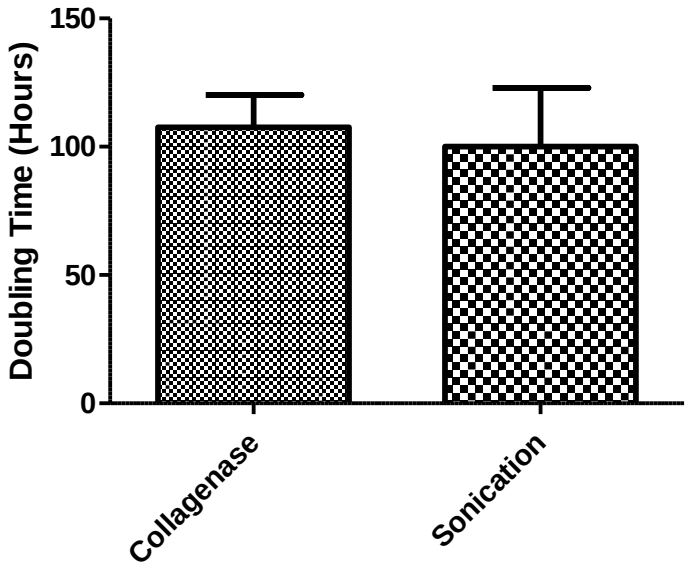


Figure 3.2. Comparison of population doubling times of adipose derived SVF cells obtained using collagenase and sonication methods. Data points represent a single measurement made from 6 samples isolated using the collagenase method and 4 samples using the sonication method +/- standard error. Samples taken from passage 1 to passage 2.

In addition to being tissue culture plastic adherent and undergoing self-replication, adipose derived MSCs are characterized by the ability to differentiate along the osteogenic, adipogenic, and chondrogenic lineages. Osteogenesis is characterized by the deposition of a calcified extra-cellular matrix which can be detected by staining with alizarin red. Similar to MSCs, the Saos-2 osteosarcoma cell line is capable of osteogenic differentiation. As positive control for alizarin red staining of calcification, Saos-2 cells were cultured for 28 days in osteogenic media or 6 days in growth media supplemented with DMSO (negative control media). Following culture, these cells were fixed and stained with alizarin red (Figure 3). Images taken of these cells indicate a strong differentiation response by Saos-2 cells cultured in osteogenic media (n=3) (Figure 3a) compared to those that received control media (n=3) (Figure 3b). The adipose SVF cells

obtained by collagenase digestion (n=6) and sonication (n=4) were similarly cultured in osteogenic and control media (collagenase n=9, sonication n=6) and subsequently stained with alizarin red (Figure 4). Compared to negative controls, cells obtained using the collagenase method from patients 2 and 3 underwent osteogenesis (Figure 4 bi-cii). However, cells which were cultured in differentiation media obtained from patient 1 displayed an absence of alizarin red staining, matching the corresponding negative control cultures (Figure ai, aii). All samples obtained using sonication stained positive for osteogenesis (Figure di-eii).

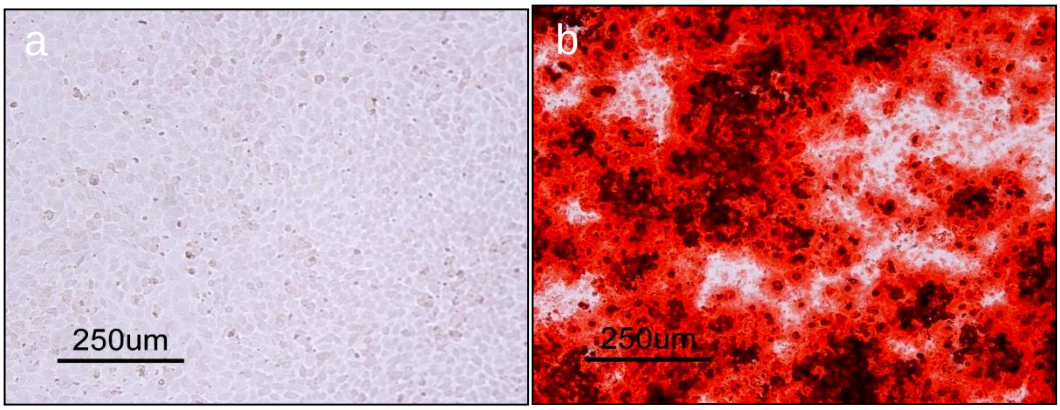
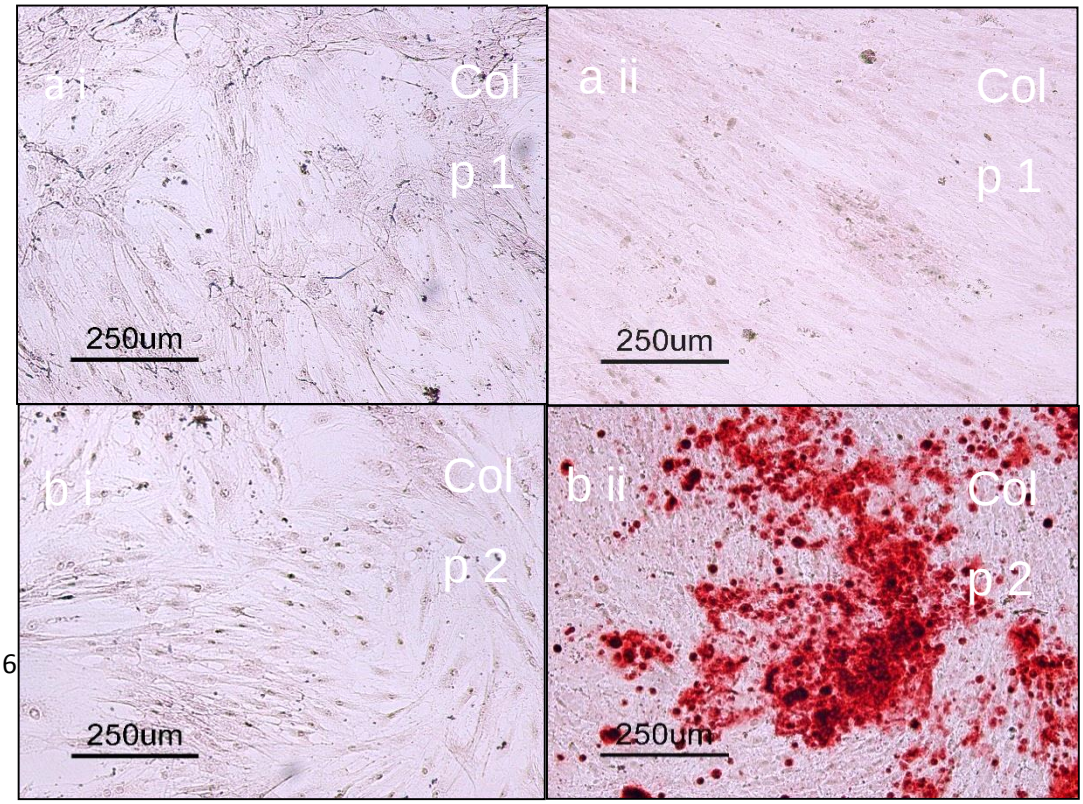


Figure 3.3. Saos-2 cells cultured in ostegenic media for 28 days (b) or control media for 6 days (a) and stained with alizarin red. Images at 10x magnification.



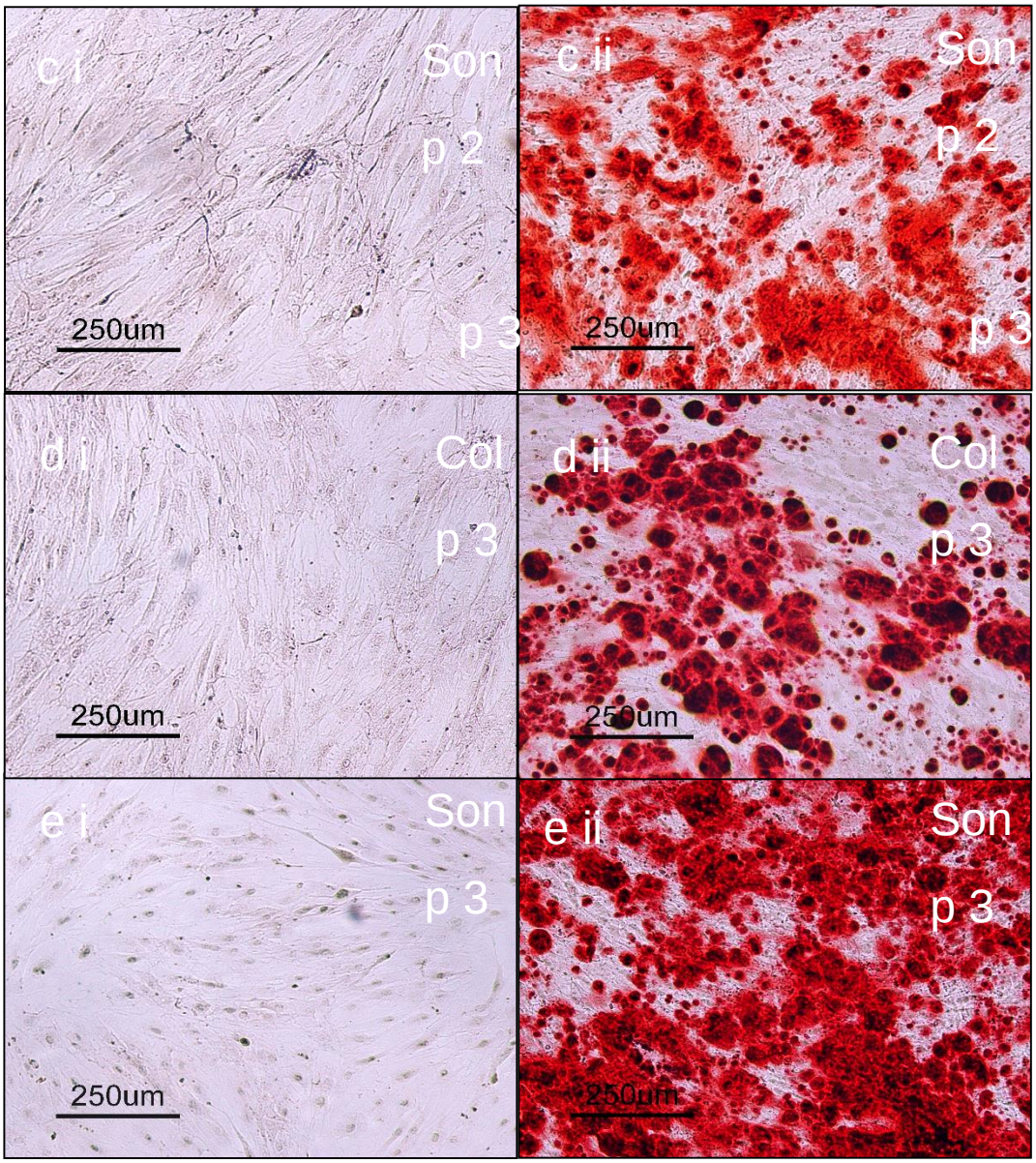


Figure 3.4. Adipose derived SVF cells cultured in ostegenic media for 28 days (aii, bii, cii, dii, e ii) or control media for 6 days (ai, bi, ci, di, ei) from patients 1-3 (p1-p3) and stained with alizarin red. Images at 10x magnification. Images a, b, and d were obtained from cells isolated using collagenase method (Col). Images c and e were obtained from cells isolated using sonication.

In order to compare the level of osteogenic differentiation between groups, the amount of alizarin red staining was assessed by eluting the dye retained by each sample. The absorbance of the dye elutant was subsequently measured at 550 nm (Figure 6). The dye elution data were analysed using the unpaired Student's T-Test with a one-tailed distribution. The collagenase, sonication, and Saos-2 treatment cultures retained significantly more dye than corresponding negative control cultures (p-values: 0.0018, <0.0001, <0.0001, respectively). Furthermore, cells isolated using sonication retained more dye than those isolated using collagenase (p-value: 0.0157). The above results demonstrate the sonication based method is capable of isolating cells with the ability to undergo osteogenic differentiation and do so to a slightly greater extent than cells isolated using collagenase.

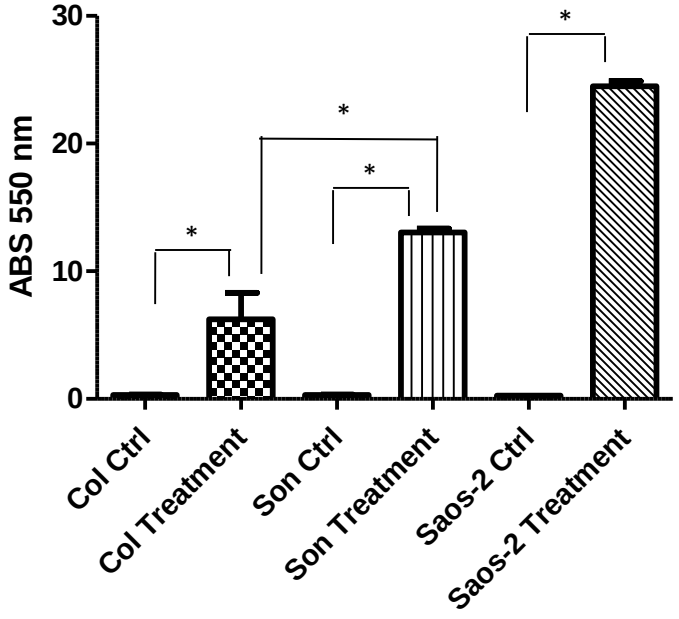


Figure 3.5. Absorbance read at 550 nm of alizarin red eluted from adipose SVF and Saos-2 cells cultured in osteogenic differentiation media (Diff) or control media (Ctrl). SVF cells were obtained using collagenase (Col) or sonication (Son) methods from patients 1-3 (P1-P3). Data

points represent measurements made in triplicate from duplicate samples, +/- standard error. Col and Son data points labelled treatment represent samples diluted in CPC at a ratio of 1:3, the absorbance values of these samples were then multiplied by a factor of 3. Saos-2 data point labelled treatment represents samples diluted in CPC at a ratio of 1:10, the absorbance values of these samples were then multiplied by a factor of 10. Individual cultures are used as replicates, where n=9 for Col treatment, n= 6 for Col control, n=6 for Son control, and n=4 for Son treatment cultures. * indicates a significant difference.

Adipogenic differentiation is characterized by the formation of spherical intracellular lipid droplets which can be detected with oil red O staining. Similar to MSCs, 3T3-L1 preadipocytes are capable of adipogenesis. As a positive control for oil red of staining of lipids, 3T3-L1 cells (n=3) were cultured in adipogenic media or negative control media (growth media supplemented with vehicle controls). Following the culture period, 3T3-L1 cells were fixed and stained with oil red O (Figure 7). Images taken of these cultures show a strong positive staining for lipids in cultures that received adipogenic media (Figure 7b) compared to those that received control media (Figure 7b). The adipose SVF cells obtained by collagenase digestion and sonication were similarly cultured in adipogenic (n=9 collagenase, n=6 sonication) and control media (n=9 collagenase, n=6 sonication) and subsequently stained with oil red O (Figure 8). Compared to negative controls, cells obtained using either collagenase (Figure 8a, b, and d) or sonication (Figure 8 c, e) from each patient displayed adipocytes stained with oil red O. However, the differentiation response of the human cells was marginal compared to that of the 3T3-L1 cells.

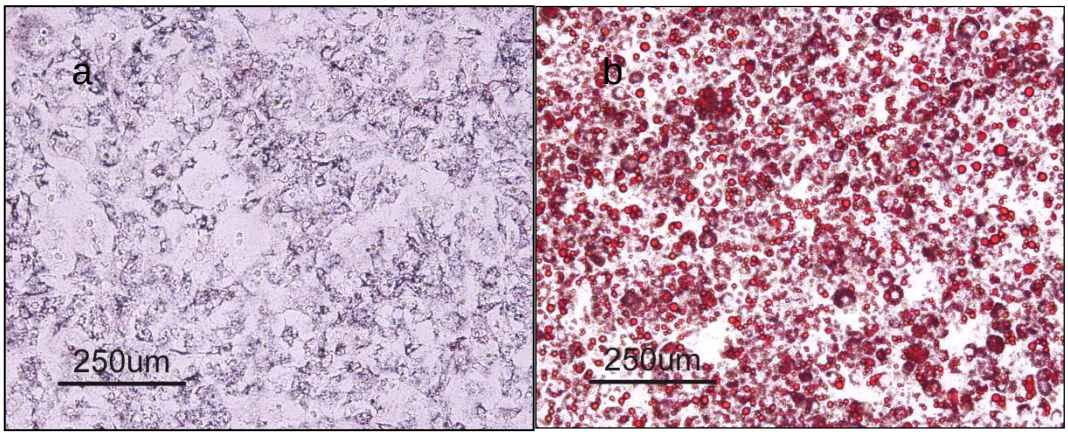
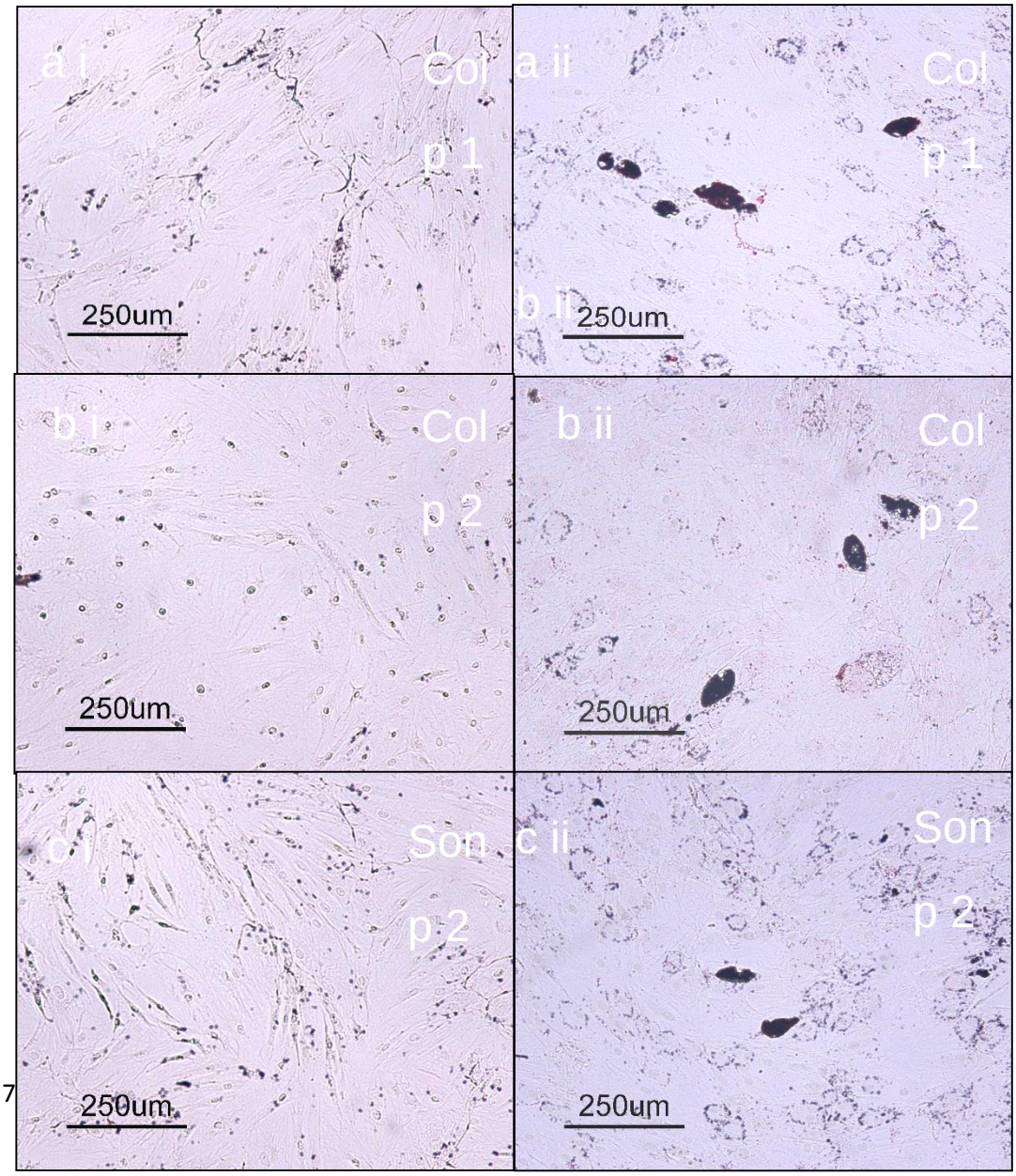


Figure 3.6. 3T3-L1 cells cultured in adipogenic media for 12 days (b) or control media for 6 days (a) and stained with oil red O. Images at 10x magnification.



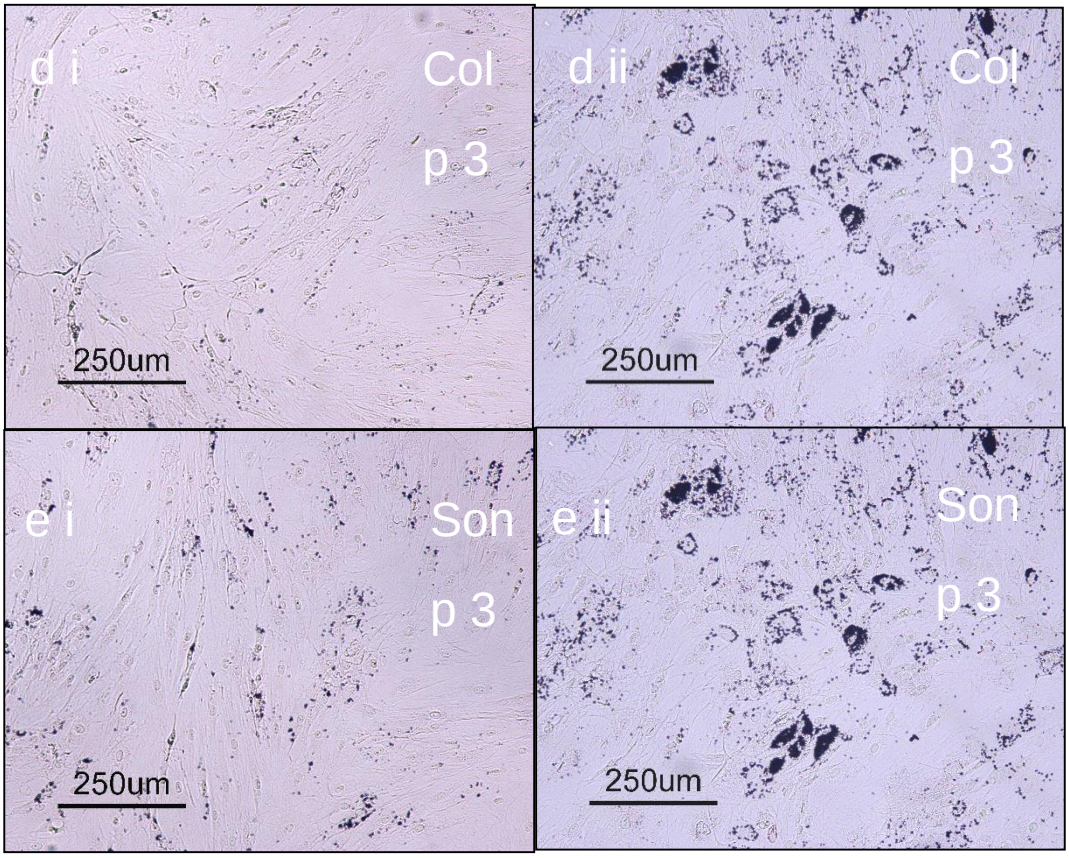


Figure 3.7. Adipose derived SVF cells cultured in adipogenic media for 12 days (aii, bii, cii, d ii, eii)or control media for 6 days (ai, bi, ci, di, ei) from patients 1-3 (p1-p3) and stained with oil red o. Images at 10x magnification. Images a, b, and d were obtained from cells isolated using collagenase method (col). Images c, and e were obtained from cells isolated using sonication (son).

In order to compare the level of adipogenic differentiation between groups, the amount of oil red O staining was assessed by eluting the dye retained by each sample. The absorbance of the dye elutant was subsequently measured at 540 nm (Figure 9). The dye elution data were analysed using the unpaired Student's T-Test with a one-tailed distribution. The collagenase, sonication, and 3T3-L1 treatment cultures retained more dye than corresponding negative control cultures, however this difference was marginal

(p-values: <0.0001, <0.0003, <0.0001 respectively). Furthermore, the amount of dye retained by cells isolated using sonication was not different compared to those isolated using collagenase (p-value: 0.8945). Together the images and dye elution show that cells isolated using sonication or collagenase experienced low levels of adipogenic differentiation.

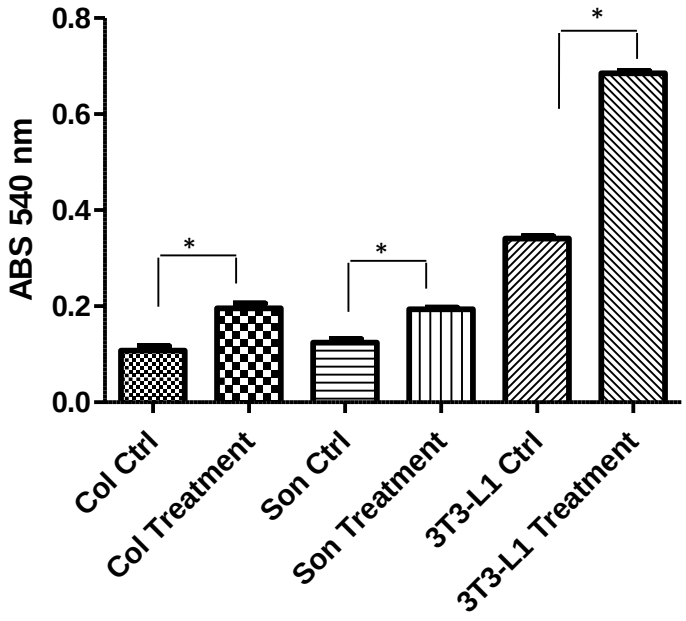
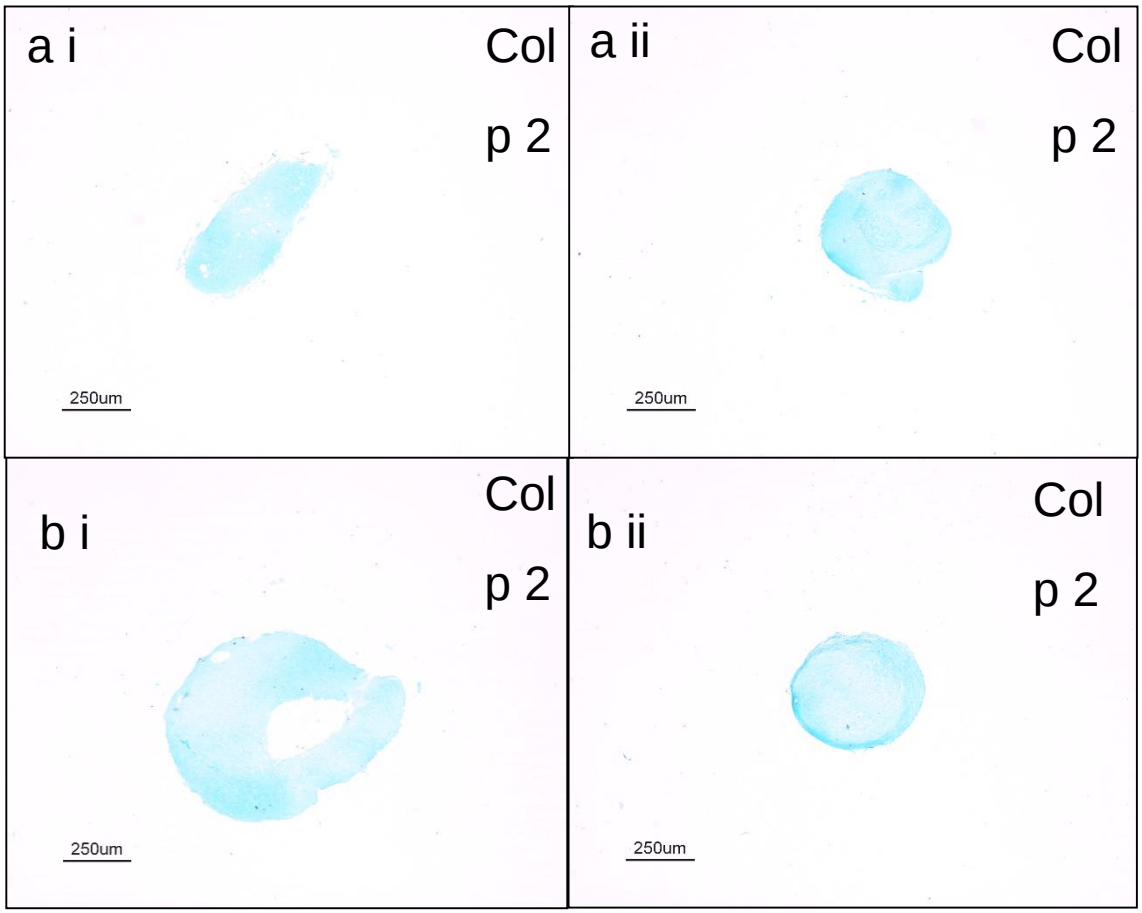


Figure 3.8. Absorbance read at 540 nm of oil red O eluted from adipose SVF cells cultured in adipogenic differentiation media (Diff) or control media (Ctrl) obtained using collagenase (Col) or sonication (Son) methods from patients 1-3 (P1-P3). Data points represent measurements made in triplicate from duplicate samples, +/- standard error. Individual cultures are used as replicates, where n=9 for Col treatment, n= 9 for Col control, n=6 for Son control,n=6 for Son treatment, and n=3 for 3T3-L1 cultures * indicates a significant difference.

Chondrogensis is characterized by the production of sulphated proteoglycans in the extra-cellular matrix which can be detected with alician blue staining. Adipose SVF cells were pelleted and cultured in chondrogenic media for 28 days or control media for 24 hrs. Following the culture period, pellets were sectioned, embedded with paraffin, and

stained with alcian blue (Figure 10). Images taken of the sections displayed no distinct difference in the levels of staining between collagenase or sonication treatment groups and their corresponding control pellets. In order to further analyse the amount of stain on the sections, ImageJ software was used to quantify the mean pixel density of sections obtained from the pellets (Figure 11). ImageJ data were analyzed using an unpaired Student's T-Test with a one-tailed distribution, $\alpha = 0.05$. A small, but significant increase with detected in staining intensity between treatment (n=5) and control (n=4) samples for pellets derived from collagenase isolated cells (p-value:0.01). No difference was detected between sonication treatment (n=6) and control (n=2) samples (p-value: 0.1466) or between collagenase and sonication treatment groups (p-value: 0.8251). The above results suggest that cells isolated using collagenase underwent a low level of chondrogenic differentiation, while those isolated using sonication did not undergo differentiation.



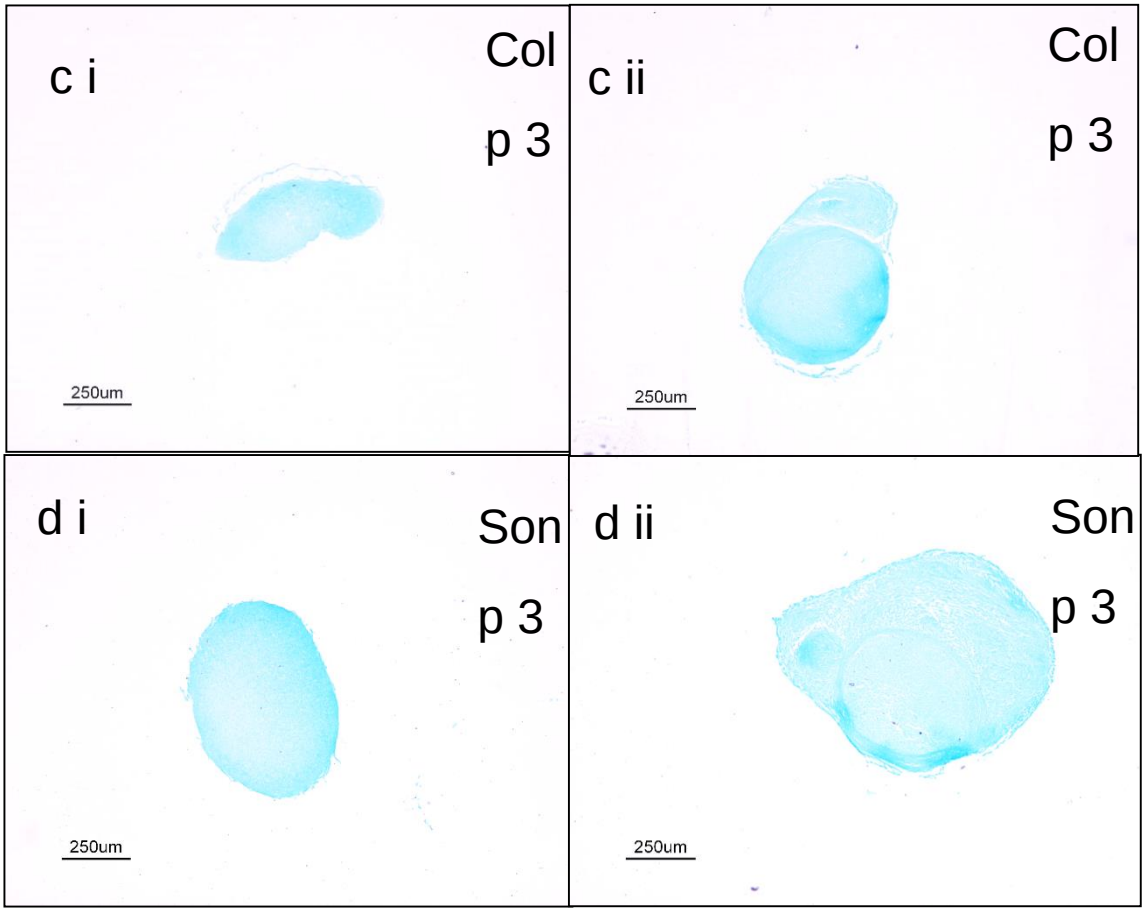


Figure 3.9. Pelleted adipose derived SVF cells cultured in chondrogenic media for 28 days (aii, bii, cii, d ii) or control media for 6 days (ai- ei) from patients 2 and 3 (p2, p3). Pelletes were embedded in paraffin, cut into 10 µm thick sections, and stained with alcian blue. Images at 4x magnification. Images a, and c were obtained from cells isolated using collagenase (col) method. Images b, and d were obtained from cells isolated using sonication (son).

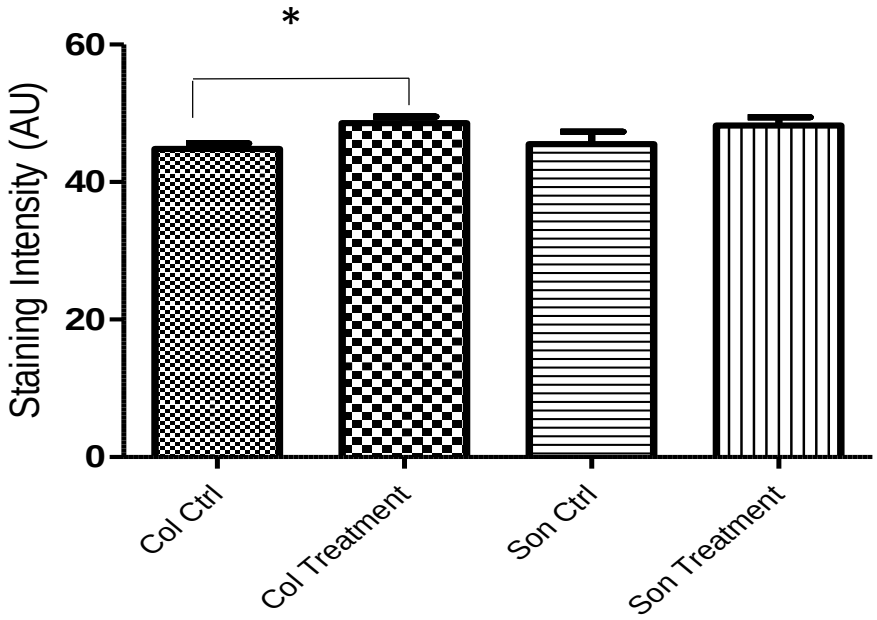


Figure 3.10. Alcian blue staining intensity of embedded cell pellet sections. Cells were isolated using either the collagenase method (col) or sonication (son) method and received chondrogenic media for 28 days (treatment) or growth media (ctrl) for 24hrs. Cell intensity was determined using ImageJ densitometry and quantified as mean pixel density in arbitrary units. Data points represent average staining intensity of sections obtained from pellets (col ctrl = 4 pellets, col treatment = 5 pellets, son ctrl = 2 pellets, son treatment = 6 pellets). Pellets were derived from cells obtained from 2 biological replicates. * indicates a significant difference.

3.4 Discussion:

The current study demonstrated that the sonication based method of cell isolation is capable of isolating proliferative, adherent cells with high ostegenic potential, but minimal adipogenic potential, and no ability to undergo chondrogenesis. Comparatively, cells isolated using collagenase digestion possessed similar differentiation capabilities, but were able to undergo a small level of chondrogenesis. Compared to the collagenase method, sonication isolated fewer passage 0 cells. Recently, Gimble et. al. developed a non-enzymatic method of isolating stem cells from adipose based on shaking lipoaspirate in PBS by hand in order to liberate cells (164). The authors observed that compared to collagenase isolation, the shaking method produced ~ 25 000 cells/ml tissue after an

average of 13 days culture time. In comparison the sonication based method produced ~ 42 000 cells/ml tissue from the two successful trials within 10 days. Similar to the shaking method, sonication yielded fewer cells than collagenase digestion. However, it is likely that the shaking method yielded fewer cells due to insufficient disruption to the adipose extra-cellular matrix. Alternatively, it is plausible that due to high energy and heat associated with ultrasonic wave emission, low cell yields through sonication may be due to excessive cell lysis.

Both collagenase and sonication derived cells underwent osteogenic differentiation. The level osteogensis as determined by alizarin red stain elution was greater in sonication derived cultures than those obtained using collagenase. There is no apparent reason why this occurred, the only difference between cultures was number of cells in passage 0 cultures. High oxygen levels have been observed to enhance osteogenesis of MSCs (165). It is possible that fewer cells in culture may have ameliorated media oxygen depletion, pre-disposing these cells along an osteogenic lineage. Nevertheless, these results suggest that the sonication method possesses potential to become incorporated into cellular therapies for bone repair.

Minimal adipogenesis was observed in sonication and collagenase derived cultures. The adipogenic treatment implemented consisted of an initial treatment period intended to induce the formation of preadipocytes from MSCs and a second treatment period which favors lipogenesis and the development of adipocytes (161). Several other adipogenic treatment protocols have been successfully used to induce a robust adipogenic response. A key inducer of adipogenesis are PPARγ agonists (107). The current protocol utilized rosglitzone, however, alternatives such indomethacin are also potent stimulators

of adipogenesis (161, 166, 88). Thus, it is plausible that with altered treatment, the isolated cells may have undergone higher levels of adipogenesis.

Following sub-culture to passage 2, isolated cells were subsequently cryopreserved, further sub-cultured and then provided treatments to induce chondrogenic differentiation. ImageJ analysis suggests that cells obtained using sonication were unable to differentiate into chondrocytes, while those obtained using collagenase underwent a small degree of differentiation. The limited chondrogenic potential of the isolated cells is likely due to over cultivation. Evidence suggests that with increased time in culture, the ability of MSCs to differentiate diminishes (88). Another possibility is that the cryopreservation process affected differentiation potential. However, research by Minonzio et. al. suggests that MSCs maintain this ability after the freezing process (167). Alternatively, several chondrogenic treatment regimes have been implemented in other studies. For example the use of bone morphogenetic proteins (BMP) and other TGFβ isoforms are alternatives to TGFβ1 used in this study (157).The results obtained from the sectioned pellets are limited due to the small number of technical replicates that were available for analysis. Thus, this study was unable to provide definitive evidence of chondrogenic differentiation. A higher sample size in addition to addition methods of assessing chondrogenesis such quantifying collagen type II and chondroitin-4-sulfate production would provide more substantial evidence (157).

3.5 Conclusions

This study was able to demonstrate that the sonication based method of isolating adipose derived SVF cells is capable of yielding proliferative, plastic adherent cells with osteogenic and adipogenic ability. These results were comparable to those obtained using

collagenase digestion, the standard method of adipose MSCs. However, ImageJ densitometry analysis of alcian blue stained sections suggests that cells obtained using sonication were unable to undergo chondrogenesis. Considering the above, it is unlikely that sonication yield multi-potent MSCs from adipose tissue. Yet, the obtained results demonstrate that the sonication method possesses potential to become incorporated into cellular therapies for bone repair.

Chapter 4: Development of a method for the differentiation of adipose-derived stem cells into brite cells

Objectives: Assess the effectiveness of different approaches to induce the differentiation of white adipose derived stromal vascular fraction cells into brite cells.

Hypothesis: Differentiation medium containing stimulators of UCP1 expression will induce stromal vascular cells to differentiate into UCP1 expressing cells with high mitochondrial content.

Abstract

Within white adipose tissue (WAT) resides populations of stem cells that can differentiate into brite cells. Similar to brown adipose tissue (BAT) cells, brite cells possess the ability to activate mitochondrial respiration via uncoupling protein 1 (UCP1)-mediated uncoupling of oxidative phosphorylation. Stimulators of UCP1 expression such as norepinephrine (NE), triiodothyronine (T_3), and rosiglitazone have been reported to induce the differentiation of WAT stem cells into brite cells *in vitro*. Considering this, the ability of media containing stimulators of UCP1 expression to induce the differentiation of WAT stem cells into brite cells was assessed here. Rat subcutaneous WAT stromal cell cultures were obtained using methods established in Chapter 2. These cells received either normal growth media or differentiation medium with or without chronic NE treatment. Cells receiving either differentiation medium developed a lipid filled morphology and high mitochondrial content as determined by citrate synthase activity. Moreover, cells that received differentiation media displayed higher oxygen consumption than those that received control media. However, neither acute NE treatment nor chronic NE treatment during differentiation significantly increased cellular respiration. The primary characteristic of brite cells is the expression of UCP1, and therefore UCP1 was probed in cell lysates using western blotting. BAT homogenates isolated from the intrascapular region of rats were used as positive controls for UCP1 detection. No UCP1 signal was detected in any lysates from stromal cells. These results suggest that while the differentiation medium utilized induced some characteristics of brite cells (lipid storage and greater mitochondrial abundance than undifferentiated control cells), differentiation was incomplete since UCP1 was absent as was NE-stimulation of respiration.

4.1 Introduction

Sedentary living and the consumption of calorie-dense food has led to a rise in obesity throughout the developed world. This is especially alarming since it is associated with the increased occurrence of obesity-associated pathologies such as hyper-tension, type 2 diabetes, hyperglycemia, and many types of cancer (135, 136). Obesity-related conditions arise due to an imbalance between energy intake and expenditure, resulting in excess energy stored as lipid in WAT (168). In addition to WAT, humans and small mammals possess stores of BAT within intrascapular regions and peripheral tissues. BAT oxidizes fuel depots in order to produce heat. This is achieved by activation of uncoupling of oxidative phosphorylation by UCP1. Because heat is produced during this process, it is called non-shivering thermogenesis (112). Furthermore, populations of stem cells within WAT are capable of differentiating into UCP1-expressing BAT-like adipocytes known as brite cells, which possess similar morphology and function to BAT adipocytes (7, 113). Investigations into the relationship between non-shivering thermogenesis and lipid metabolism have revealed that enhanced BAT activity is capable of conferring anti-obesity properties. For example, UCP1-knockout mice housed under thermo-neutral conditions ($30^{o}C$) gained more weight within adipose depots than wild-type controls (114) Moreover, Kopecky *et al*. 1995 constitutively expressed UCP1 in WAT depots of genetically obese A^{vy} mice. Compared to non-transgenic mice, UCP1 expressing mice displayed reductions in total body weight and subcutaneous depots. (137). Thus, the induction of brite cell differentiation from WAT derived stem cells is a potential therapeutic intervention to ameliorate the symptoms of obesity-associated diseases. In theory, one could harvest these WAT derived stem cells in the aspirate from

a liposuction patient, differentiate them into brite cells, and re-introduce them into the patient to promote long-term energy homeostasis and ameliorate weight gain.

In order to induce the differentiation of WAT derived SVF stem cells into brite cells *in vitro*, specific culture supplements have been utilized. For example, NE, rosiglitazone, and T_3 activate the UCP1 promoter region, causing increased UCP1 transcription (7, 112, 113, 118-120,124). WAT SVF cells or brown pre-adipocytes cultured in the presence of these inducers differentiate into brite cells and BAT cells, respectively. In addition to specific stimulators of UCP1, IBMX in combination with insulin and dexamethasone is commonly utilized to induce brite cell and brown adipocyte differentiation. IBMX is hypothesized to indirectly increase UCP1 transcription by elevating cAMP levels (115). Insulin and dexamethasone (a synthetic glucocorticoid) are also necessary for brite cell differentiation. Little is known concerning the specific molecular mechanisms of these molecules in the differentiation process. However, the presence of glucocorticoids is postulated to be necessary for the accumulation of lipid stores in differentiating brown adipocytes (123). Moreover, the presence of insulin and the activation of its downstream effector, IRS, are necessary for brown adipogenesis (125). Considering the above, the effectiveness differentiation medium containing NE, T_3, indomethacin, rosiglitazone, dexamethasone, IBMX, and insulin to induce a brite cell phenotype in WAT SVF cells was assessed.

4.2 Materials and Methods

Acetyl CoA, 3-isobutyl-1-methylxanthine (IBMX), bovine serum albumin (BSA) fraction V, dexamethasone, d- pantothenate, dimethyl sulfoxide (DMSO), 5,5'-dithiobis

(2-nitrobenzoic acid), dithiolthreitol, fetal bovine serum (FBS), human recombinant insulin, norepinephrine, oxaloacetate, paraformaldehyde, penicillin/ streptomycin, rosiglitazone, 0.25% trypsin-EDTA, and triiodothyronine were purchased from Sigma Aldrich.

DMEM/ F-12 powdered media with L-glutamine, and Ham's F-12 powdered media with L-glutamine were purchased from Invitrogen. Collagenase type I was purchased from Worthington Biochemical. Hydrochloric acid and sodium hydroxide were purchased from Caledon.

Potassium chloride, potassium diphosphate, monobasic potassium phosphate, sodium bicarbonate, sodium chloride, dibasic sodium phosphate, and Tris were purchased from Fischer Scientific. Glycerol, NP-40, and triton X-100 were purchased from Bioshop Canada. Bradford assay kit was purchased from Biorad. Polyvinylidene difluoride and rabbit anti-UCP1 antibody were purchased from Abcam. Rabbit anti-MnSOD antibody was purchased from Stressgen. Rabbit anti-actin antibody was purchased from Santa Cruz. Anti-rabbit IR800 antibody was purchased from Rockland.

4.2.1 Animals and primary cell line isolation

The use of experimental animals was approved by the Brock University ACUC. Young adult Long Evans rats were euthanized by decapitation with a guillotine. Subcutaneous WAT and intra-scapular BAT were excised and subjected to collagenase digestion as per methods 2.2.3. Digested brown adipose tissue was stored at -80^{o}C.

4.2.2 Cell Culture:

Stromal vascular cells obtained from WAT were cultured in growth media at 37˚C, 5% CO_2 and 3% O_2. Once passage 0 cultures reached ~70-80% confluence the cultures were split at a ratio of 1:2 using 0.25% trypsin-EDTA solution. Adipose derived cells were further subcultured for 1-2 more passages until confluency was reached. Subsequently, cells were exposed to trypsin and plated on 35 mm tissue culture treated glass bottom dishes at a density of 15 000 cells/cm^2. Once reaching confluency cells were transferred to a 20% O_2 incubator and were treated with differentiation treatments or corresponding vehicles.

Brite Cell Differentiation Treatment: For 48 h cells were cultured in induction media consisting of growth media supplemented with 5µg/ml insulin (dissolved in 4mM HCl), 1nM triiodothyronine dissolved in 1M NaOH, 125uM indomethacin dissolved in ethanol, 2µg/ml dexamethasone dissolved in ethanol, 0.5mM IBMX dissolved in DMSO, and 0.5µM rosiglitazone dissolved in DMSO. For an additional 48h, cells received maintenance media consisting of growth media supplemented with 5µg/ml insulin, 1nM triiodothyronine, and 0.5µM rosiglitazone. Subsequently, cells were then cultured in a second maintenance media consisting of 5µg/ml insulin, 1nM triiodothyronine, and 1µM rosiglitazone for 5 days. During the final 24h of treatment a separate group of cells also received 1µM norepinephrine (NE). Additionally, cultures of control cells received equivalent volumes of the vehicle solvents provided to cells receiving differentiation medium. For 48h cells received growth media supplemented with $1x10^{-4}$ % 4mM HCl, $1x10^{-5}$ % 1M NaOH, $2x10^{-4}$ % ethanol, and $2x10^{-4}$ % DMSO. For additional 48h, control cells received growth media supplemented with $1x10^{-4}$ 4mM HCl, $1x10^{-5}$ % NaOH, and

$5x10^{-6}$ % DMSO. Control cells then received the same supplemented growth medium for 5 days except with $1x10^{-5}$ % DMSO.

4.2.3 Imaging:

Phase contrast images of cells in culture were captured using the Hund Wilovert S inverted microsocope from Fischer Scientific.

4.2.4 Cellular Respiration:

Cellular oxygen consumption was measured at 37ºC using a Rank Brothers Dual digital (model 20) respirometer with a water-jacketed cell chamber. Treated and control cells were washed with PBS, harvested from culture plates using trypsin, centrifuged at 700 *x g* for 2min, and resuspended in 1ml of DMEM/F-12 without FBS. The cell suspension was then added to the respirometry chamber. Respiration rates of cells were recorded for approximately 10 min. Respiration rates were determined once a stable linear rate was obtained and again following introduction of 1µM NE to respiring cells. Subsequently, cell suspensions were collected from the cell chamber, washed with PBS, pelleted by centrifugation at 700 *x g* for 2min and frozen at -80ºC.

4.2.5 Preparation of lysates from WAT SVF cell and Brown adipose tissue:

Lysates were prepared from frozen white adipose-derived cell pellets and digested BAT. Cells and tissue digests were resuspended in two volumes of ice cold lysis buffer (10mM Tris pH 8.0, 150mM NaCl, 2mM EDTA, 2mM Dithiolthreitol (DTT), 40% glycerol, and 0.5% NP40) and incubated for 1hr with periodic sonication (Ultrasonic Inc Sonicator W-375). Following incubation, cell lysates were centrifuged at 10 000 *x g* at 4ºC for 10min (Fisher Scientific accuSpin™ Micro R). Protein concentrations of the cell

and tissue lysates were determined by the Bradford method using a BioRad protein assay kit. Lysates were stored at -80^{o}C.

4.2.6 Citrate Synthase Activity:

Citrate synthase activity was determined using a Varian Cary 100 Bio UV-Visible Spectrophotometer equipped with a Peltier thermostatable cell changer maintained at 30^{o}C. Citrate synthase was monitored at 412nm. The assay buffer contained 50mM Tris pH 8.0, 0.5mM, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1mM Acetyl CoA, 0.05% Triton X-100, and 20µg protein. The reaction was initiated by the addition of 0.5mM oxaloacetate and absorbance was followed for approximately 2min.

4.2.7 SDS PAGE and Western Blot:

The presence of UCP1 in cell lysates was assessed using western blot analysis. MnSOD was used as a proxy of mitochondrial abundance. Equal amounts (50 ug) of whole cell or BAT lysates were subjected to SDS-PAGE on 4% stacking, 12% resolving gels and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were visualized using the Odyssey (Li-Cor, Lincoln, NE) detection system. Membranes were probed with rabbit anti-MnSOD (1:400 dilution) and rabbit anti-UCP1 (1:250 dilution) primary antibodies. Subsequently, membranes were probed with IR 700 conjugate anti-rabbit secondary (1:2500 dilution). Images were manipulated and analyzed using Odyssey imaging software 1.0.

4.2.8 Statistical Analysis:

Oxygen consumption and citrate synthase activity data were analyzed using the Student's T-Test with a one tailed distribution on GraphPad Prisim version 5. $\alpha = 0.05$ was used to determined statistical significance.

4.3 Results:

In order to induce brite cell differentiation in SVF cells, cultures were incubated in differentiation media (see materials and methods 4.3.3) for a period of 9 days. Since norepinephrine (NE) is a potent stimulator of UCP1 expression (124) a separate group of SVF cells received 1µM NE (chronic treatment) in addition to brite cell differentiation media during the final 24h of culture. Both differentiation regimes promoted the development of a phenotype characterized by the accumulation of abundant lipid droplets within the cytoplasm. (Figure 4.1).

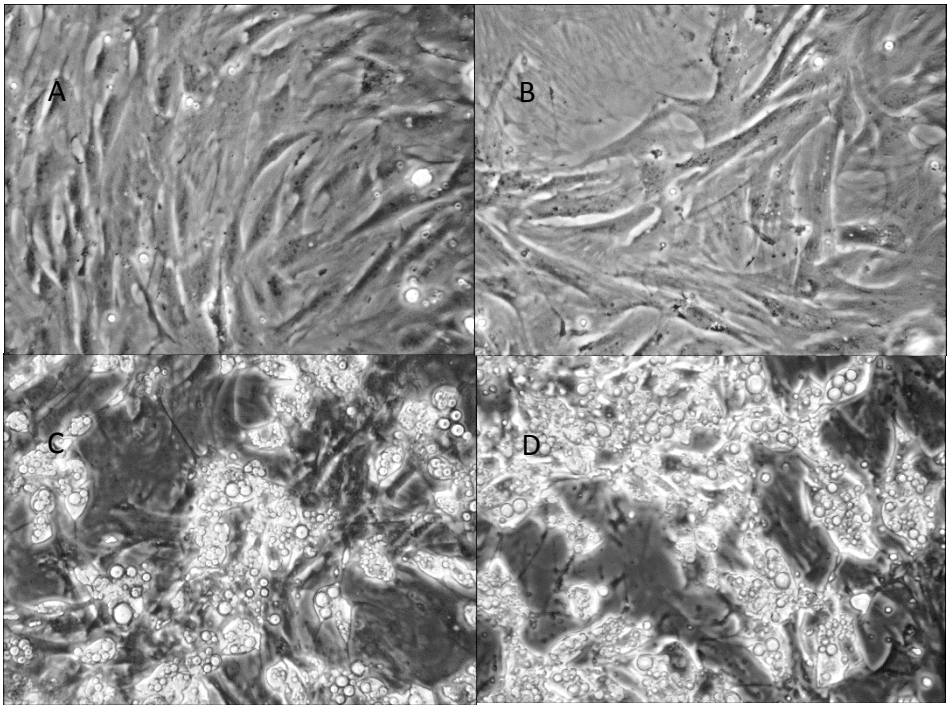


Figure 4.1 Phase contrast images of adipose derived SVF cells following Brite cell differentiation treatment with and without chronic NE treatment. Images obtained from at day 0 of treatment (a, b) and at day 9 (c,d). Image d is of cells exposed to chronic NE during the final 24h. Images taken at 20x magnification.

In addition to inducing UCP1 expression, NE also stimulates UCP1 activity and therefore increases cellular O_2 consumption (7, 113). To determine if the treated cells possessed NE-sensitive uncoupling capacity, the rate of oxygen consumption of cells (n=5) treated with differentiation media was monitored before and after acute 1µM NE treatment (Figure 4.2). No significant difference in oxygen consumption was observed after NE treatment (p-value 0.2519; paired T-test). Cultures that received chronic NE treatment prior to acute treatment displayed a small but not quite significant increase in the rate of O_2 compared to cultures that received control media before (p-value 0.0581; T-test) and a significant increase after acute NE treatment (p-value 0.0133). However acute NE treatment did not stimulate O_2 consumption of cells cultured in the presence of differentiation media (p-value 0.9321) (Figure 4.3).

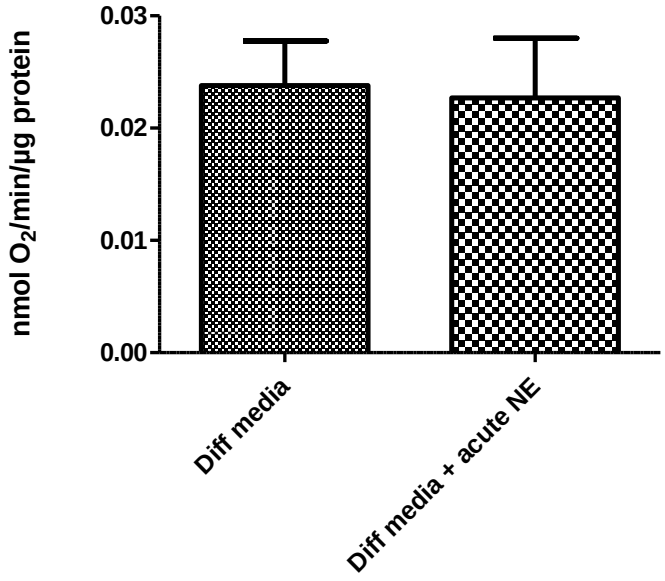


Figure 4.2 Respiration rates of adipose SVF cells cultured in brite cell differentiation medium before and after acute 1µM NE. Data points represent measurements from 2 to 3 technical replicates obtained from 5 biological replicates, +/- standard error. N=5 for biological replicates.

In addition to possessing thermogenic activity, the brite cell phenotype is characterized by increased mitochondrial abundance (124). Furthermore, the activity of the mitochondrial matrix enzyme citrate synthase is a strong indicator of mitochondrial abundance (169). In order to determine if more mitochondria were present in cells cultured in brite cell differentiation medium, the specific activity of citrate synthase was spectrophotometrically monitored at 412nm (Figure 4.4). Cells cultured in the presence of differentiation medium (n=5) with and without chronic NE displayed higher citrate synthase activity (p-values 0.0105, and >0.0001, respectively) compared to cells cultured in control media. Additionally, no difference in citrate synthase activity was observed between cells that received chronic NE and those that received differentiation media alone (p-value 0.4829). Taken together, the above results suggest that the differentiation treatment induced SVF cells to produce more mitochondria, suggesting that a brite cell phenotype may be present.

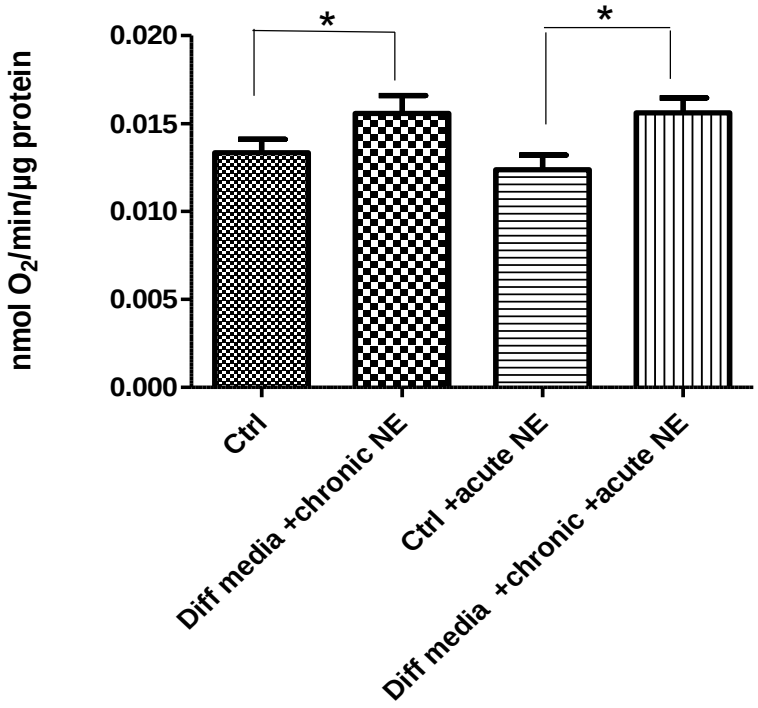


Figure 4.4 Citrate synthase activity monitored at 412nm of adipose SVF cells cultured in brite cell differentiation medium (diff) in presence or absence of chronic (24hr) 1µM NE or control (ctrl) medium. Data points represent measurements from 2 to 3 technical replicates obtained from 5 biological replicates, +/- standard error. * indicates a significant difference.

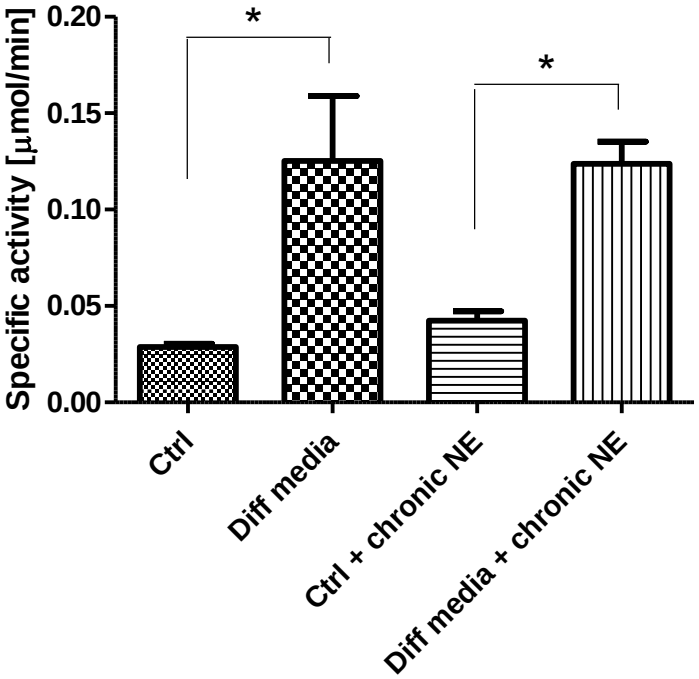


Figure 4.3 Respiration rates of adipose SVF cells cultured in brite cell differentiation (diff) medium in presence or absence of chronic (24hr) NE treatment before and after acute 1µM NE or control (ctrl) medium. Data points represent measurements from 2 to 3 technical replicates obtained from 5 biological replicates, +/- standard error. * indicates a significant difference.

The primary distinguishing characteristic of brite cells is the expression of UCP1. Therefore the presence of UCP1 in SVF cells was assessed using western blotting. In addition to brite cells, brown adipocytes are only cell type that expresses UCP1. Thus, BAT protein extracts were used as a positive control for UCP1 detection. Lysates obtained from BAT and cultures exposed to chronic NE treatment (2 biological replicates) were probed for UCP1 (Figure 4.5 and Appendix Figure 2). UCP1 was only observed in lanes containing BAT lysate. Thus, above evidence suggests that the differentiation medium did not induce WAT SVF cells to differentiate into brite cells.

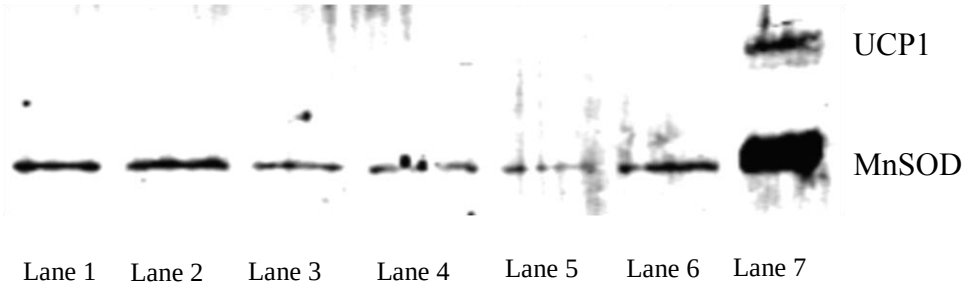


Figure 4.5 Representative western blot for the presence UCP1 and MnSOD in lysates obtained from adipose SVF cells cultured in brite cell differentiation medium for 9 days with chronic (24hr) 1μM NE or control medium. Lanes 1, 3, and 5 display protein obtained from control cells. Lanes 2, 4, and 6 display protein obtained from cells cultured in differentiation medium. Lane 7 displays protein obtained from BAT extract.

4.4 Discussion

Cultures that received either differentiation media or differentiation media plus chronic NE developed a lipid filled morphology following 9 days of treatment. This characteristic is associated with BAT adipocytes, brite cells, and WAT adipocytes (96, 112, 113). A possible explanation is that dexamethasone (a synthetic glucocorticoid receptor agonist) within the differentiation media induced this morphological change. Li *et al.* 2006 observed that dexamethasone treatment alone increases the level of lipid

accumulation within cultured marrow MSCs (170). Prior to lipid accumulation, WAT stromal cells displayed a fibroblast-like phenotype, suggesting that the differentiation medium induced stromal cells to possess an adipogenic phenotype.

Compared to undifferentiated stem cells and WAT adipocytes, brite cells and brown adipocytes contain high amounts of mitochondria in order to generate heat via thermogenesis (112, 113, 171). In order to assess mitochondrial abundance, citrate synthase activity was measured. The citrate synthase activity of cells cultured in differentiation medium (with and without chronic NE) was higher than that of control cultures, indicating an increased mitochondrial content in treated cells. Petrovic *et al.* 2010 observed that WAT stromal cells induced to differentiate into brite displayed increased expression of PGC1α, a marker of mitochondrial content (124). Considering that WAT possesses a low mitochondrial content, the above results suggest that the differentiation medium induced the WAT SVF cells to develop more mitochondria and produce lipid droplets, features that are consistent with differentiated brite cells.

Previous investigations have demonstrated that acute NE exposure increases respiration in BAT and brite cells by activating UCP1 dependent thermogenesis (7, 113, 124). Thus, in order to assess if the differentiation medium induced differentiation of WAT SVF cells into brite cells, the rate of oxygen consumption was measured before and after acute NE exposure. Cultures which received differentiation medium plus chronic NE had a higher rate of respiration compared to negative controls. Considering that mitochondrial content is proportional to the amount of oxidative phosphorylation a cell can undergo, this result agrees with the observation that cells which received chronic NE also displayed higher citrate synthase activity. Furthermore, cultures that received

differentiation medium or differentiation medium plus chronic NE did not display increased respiration following acute NE exposure. Considering that UCP1 is required to elicit this effect, the above result suggests that the differentiation medium did not induce UCP1 expression and therefore brite cell differentiation. A possible reason that acute NE had no effect is that treated cells did not express β_3-adernonergic receptors required to activate UCP1. In addition to NE, fatty acids such as oleic and linoleic acids directly activate UCP1 (171). Considering this, it would be worthwhile to evaluate O_2 consumption of treated cells in the presence of fatty acids instead of NE.

In order to determine if the differentiation medium induced UCP1 expression, cell lysates were probed for the presence of UCP1 using western blotting. Positive UCP1 bands were only detected in BAT extracts run alongside cell lysates obtained from cultures exposed to chronic NE, therefore results obtained from cells treated with differentiation medium alone are not reliable. Although UCP1 was detected in BAT, no corresponding UCP1 expression was observed in cells that received differentiation medium plus chronic NE. Taken together, the above results suggest that the differentiation medium induced WAT SVF cells to develop intracellular lipids and increased mitochondrial content. Yet, NE induced thermogenesis and UCP1 expression were absent in these cells. Thus, it seems that there may have been partial differentiation and a brite cell type was not fully achieved. The brite differentiation medium and treatment regime utilized was based the protocol published by Aune et al. 2013 (119). However, little consistency is present in regards to a unified method of differentiating WAT SVF cells to brite cells. For example, the number of differentiation inducers included and concentrations of constituents within differentiation mediums varies greatly

between research groups (117-120,124). Additionally, treatment of WAT stromal cells with rosiglitazone or T_3 alone has been shown to induce brite cell differentiation (117). Moreover, stromal cells were obtained from subcutaneous WAT depots as in Aune et al. However, brite cell differentiation has also been achieved in stromal cells obtained from WAT surrounding the epididymis (124). Thus, it is possible that varying the levels of the compounds used within the differentiation medium or treating epididymally derived WAT stromal cells may have resulted in UCP1 expressing brite cells.

4.5 Conclusions

This set of experiments showed that the brite cell differentiation method utilized was capable of producing cells with high mitochondrial content and abundant stored lipid droplets. However, the medium did not induce UCP1 expression or a capacity for NE-stimulated respiration. Future efforts to differentiate WAT stromal cells into brite cells should assess differentiation medium containing fewer constituents such as using T_3 or rosiglitazone alone, and utilizing epididymal WAT stromal cells instead of subcutaneous WAT cells.

Chapter 5: General Discussion

5.1: MSCs isolated using sonication

Methods for obtaining MSCs from adipose have traditionally used collagenase to isolate stromal cells from the surrounding extracellular matrix. However, this method is not ideal for clinical applications because the sample will be contaminated with potentially toxic exogenous enzyme that cannot enter a patient. Here it was shown that adipose-derived stromal cells can be isolated using ultrasonic waves emitted from a tissue sonicator, eliminating the need for enzyme. In order to be applicable to regenerative

therapies for age-associated diseases, the sonication method must be capable of yielding enough cells to administer into a patient and yield cells possessing the ability to differentiate into specialized cell types. Investigations using MSCs for the regeneration of bone defects have utilized $2.5x10^5 – 7.5x10^6$ cells (128-131) and $10x10^6 – 40x10^6$ cells for cartilage defects (132, 133), depending on the size of the defect and model organism. Following the first passage *in vitro,* an average of $1.18x10^6$ stromal cells were isolated from adipose of 2 patients using the sonication method. In comparison the, collagenase method yielded $2.93x10^6$ cells. It is plausible that sonication yielded less cells because the high levels of heat associated with sonication caused cell death. Thus, a potential means to increase cell yields using sonication would be to lower the ultrasonic exposure time and/or decrease ultrasonic wave intensity. Nevertheless, in order to obtain enough adipose-derived MSCs for tissue regeneration, the Regenastem sonication method will likely require additional *in vitro* passage steps to increase the total number of cells obtained.

In addition to cell yield, stromal cells must possess the ability to differentiate in order to facilitate tissue regeneration. Adipose stromal cells isolated using sonication displayed a robust ability to undergo osteogenic differentiation, but not chondrogenic differentiation. The osteogenic capability of these cells gives the sonication method potential for orthopedic applications. However, the inability to undergo chondrogenisis limits the usefulness of the cells isolated with sonication to repair cartilaginous tissue. Moreover, this limited differentiation potential raises the question if the sonication method yielded any MSCs. Stromal cells isolated using collagenase did show limited chondrogenic potential in addition to limited adipogenic and osteogenic potential,

suggesting that MSCs were present in the intact adipose. Considering this, it is plausible that more alternate chondrogenic differentiation methods may have induced a response in the cells obtained using sonication. For example, culturing MSCs compressed within polymer beads has been demonstrated to enhance chondrogenic differentiation (10). Moreover, inter-donor variability in MSC sensitivity to specific differentiation inducers has been reported (10). Thus, using alternate inducers such as other TGFβ isoforms and BMPs may elicit a chondrogenic response from stromal cells isolated using sonication.

5.2: Adipose Stromal Heterogeneity

The stroma of adipose contains multiple cell types in addition to MSCs (33). Thus, when adipose MSCs are isolated for therapeutic applications, a heterogeneous population of cells will be obtained. Because MSCs represent a small fraction of total stromal cells, adipose stromal suspensions diluted with non-MSC cell types may possess limited potency of a MSC based therapy. Here, adipose and marrow derived stromal cells were incubated with anti-CD 54, 31, and, 45 antibody conjugated beads to facilitate the removal of fibroblasts, endothelial cells, and immune cells, respectively. It was demonstrated that this enrichment procedure had no effect on the differentiation capacity of stromal cells. These results suggest that purification prior to introducing MSCs into a patient may not be necessary. This concept is supported by *in vivo* studies that have demonstrated tissue regeneration using non-purified stromal cell suspensions (128, 131). Moreover, it is possible that non-MSC cell types may aide in the MSC differentiation process, and therefore should remain in stem cell suspensions. This hypothesis is supported by studies using MSC co-culture systems. For example Kaigler et al. 2005 cultured endothelial cells with marrow MSCs (173). The *in vitro* study showed that the

osteogenic ability of MSCs increased when in direct contact with endothelial cells. *In vivo,* the authors showed that when endothelial cells combined with MSCs increased bone formation compared to fibroblast and MSCs. Similar results have been observed autologous derived MSCs in rats and goats (174, 175). Moreover, in order to obtain sufficient numbers of cells to implement magnetic sorting cells were stromal cells were expanded for 10 passages. Other groups have observed that *in vitro* expansion limits adipogenic and osteogenic differentiation potential (153, 154). Thus it is plausible that prior to the enrichment procedure, the differentiation potential of stromal cells was diminished. With this in mind, non-enzymatic isolation methods (e.g. sonication) and enrichment procedures should limit *in vitro* passaging prior to utilizing MSCs for regenerative applications.

5.3: Brite cell differentiation

The biomedical interest in brite and brown adipocytes has focused on the capacity of these cell types to counteract diseases such as obesity and diabetes. Such interest stems from studies demonstrating that increased activity of brite and brown adipocytes is linked to obesity resistance in mouse models (12, 13). Recruitable populations of brite cells reside within WAT depots and are believed to originate from resident MSCs (7, 113). Thus an important step in utilising brite cells to combat obesity in humans is the development of *in vitro* models of brite cell differentiation. Here, adipose stromal cells were incubated with known stimulators of UCP1 expression and brown adipocyte differentiation in order to induce brite cell differentiation. Following treatment, cultures displayed characteristics of the brite cell phenotype such as increased mitochondrial content and lipid accumulation. However, the treated cells were not capable of NE

induced thermogenesis or UCP1 expression. Thus, the differentiation protocol utilized was unable to induce brite cell differentiation. In order to achieve this, potential adjustments to the differentiation method can be implemented. The treatment regime (see chapter 4, page 98) relies on the initial induction of stromal cells along the adipogenic lineage (119). Subsequent to this, cells received different cocktails to induce a brite cell phenotype. Thus it is plausible that extending induction or differentiation treatment phases may produce brite cells. Moreover, several groups have utilized the same inducers as used here (117-121), but at different concentrations. It would be worthwhile to determine if altering the concentrations of media constituents would result in differentiation.

6: Conclusions

Adipose derived MSCs possess the ability to differentiate into specialized cell types capable of ameliorating symptoms of several age-associated diseases. Moreover, adipose is an ideal source of stem cells due to its abundance and accessibility. Thus developing novel methods of isolating and differentiating adipose-derived MSCs is an important step in creating efficient and minimally invasive regenerative therapies. During any isolation procedure for obtaining adipose MSCs, a heterogeneous population of adipose stromal cells will be obtained. The use of cell populations in which MSCs are significantly diluted by non-MSC stromal cells may limit the capacity of MSCs to differentiate *in vitro* and potentially their ability to facilitate tissue repair. Considering this, a magnetic-bead based method of enriching adipose stromal cell cultures was assessed. Cells were incubated with magnetic beads targeted at fibroblasts, endothelial cells, and immune cells. No significant differences were observed in the ability of

enriched cultures to differentiate compared to cultures that were subjected to enrichment. This observation suggests that the removal of non-MSC stromal cells may not impact the ability of MSCs to differentiate. However, it is likely that prolonged time in culture affected the ability of these cells to differentiate. Thus, future efforts to enrich adipose stromal cultures should minimize pre-enrichment culture time.

In order to eliminate the need for digestive enzyme in obtaining adipose MSCs, a sonication based method of MSC isolation was assessed. The sonication based method of isolating adipose stromal cells was capable of yielding proliferative, plastic adherent cells with osteogenic and adipogenic ability. However, cells isolated using sonication were not unable to differentiate into chondrocytes. Considering the above, it is unlikely that sonication yielded multi-potent MSCs from adipose tissue. However, it is plausible that a medium containing alternate stimulators of chondrogenisis may have induced differentiation. Considering this, future work assessing the sonication method should implement growth factors such as BMPs or other TGFβ isoforms within chondrogenic medium.

Lastly, an *in vitro* method of inducing adipose derived stromal cells to differentiate into brite cells was assessed. The brite cell differentiation medium utilized was capable of causing cells to have a high mitochondrial content and the produce of lipid droplets. Yet, the medium did not induce the differentiation of WAT SVF cells into UCP1 expressing cells capable of NE induced non-shivering thermogenesis. Therefore, the differentiation method did not induce SVF cells to become brite cells. The treatment regime used known inducers of UCP1 expression and brown adipocyte differentiation. Future efforts based on this treatment regime may assess whether extending treatment

induction/differentiation periods with these inducers or altering the concentrations of inducers is capable of producing brite cells.

7. Appendix:

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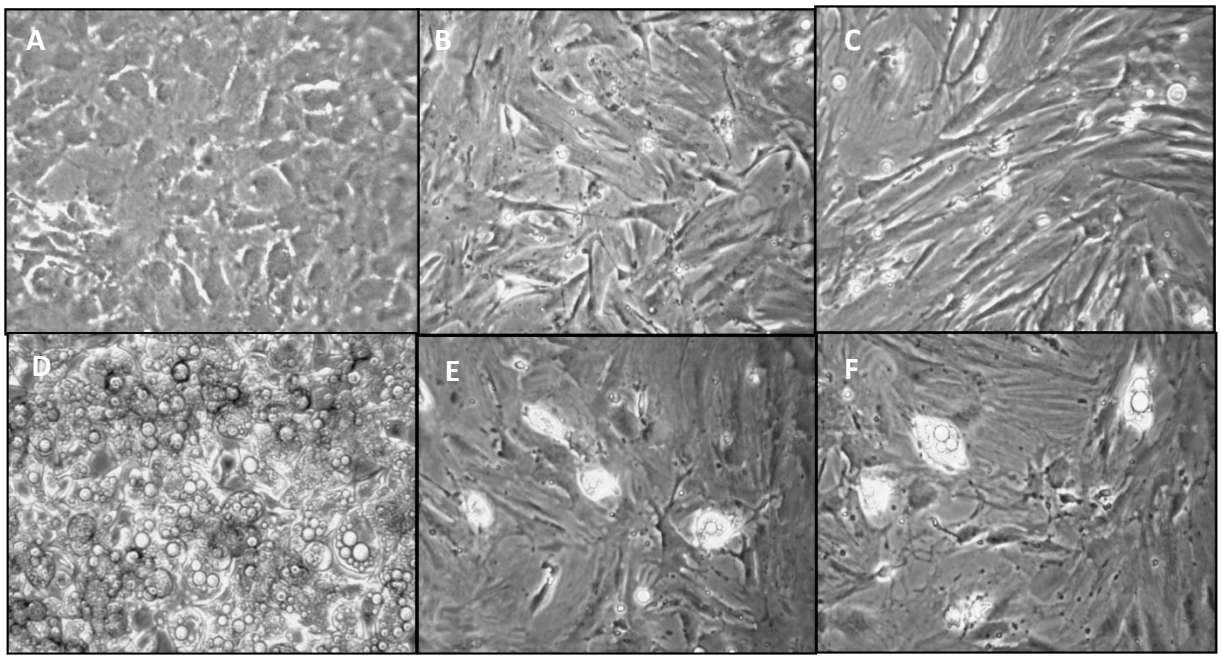
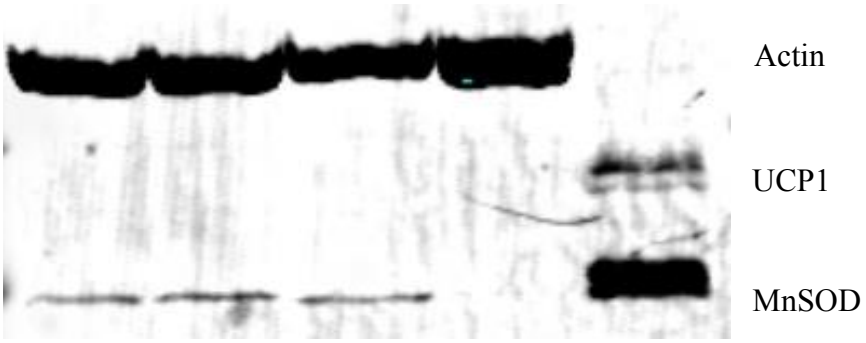


Appendix Figure 1. Phase contrast images of adipose SVF cells following magnetic isolation and adipogenic treatment. Adipose SVF cells were subjected to magnetic separation (c, f) or excluded from the separation procedure (b, e). 3T3-L1 cells (a, d) shown as positive control. Cells received either adipogenic medium for 12 days (d, e, f) or growth medium supplemented with vehicle for 6 days (a, b, c). Images taken at 20x magnification.



Appendix Figure 2. Representative western blot for the presence of UCP1, MnSOD, and actin in lysates obtained from adipose SVF cells cultured in brite cell differentiation medium for 9 days with chronic (24hr) 1μM NE or control medium. Lanes 1, 2, and 3 display protein obtained from cells cultured in differentiation. Lane 4 display protein obtained from cells cultured in control medium. Lane 5 displays protein obtained from BAT extract.

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